fast red violet salt (Sigma Chemical Co.) and incubated at 37 °C for 120 min. After the solution was removed by washing, specimens were counterstained with hematoxylin and observed under light microscopy.

2.10. Computed tomography (CT) scanning images

Computed tomography (CT) data on harvested distal femurs at 24 weeks was collected with a helical CT (GE Yokogawa, Tokyo, Japan) and images were reconstructed using 3-dimensional image reconstruction software (Aze, Tokyo, Japan).

2.11. Statistical analysis

Student's t-test was used to determine statistical significance, with $P < 0.05$ considered significant.

3. Results

3.1. Defect repair effect of rhBMP-2/PLA-DX-PEG/β-TCP (Study 1)

Representative radiology of bone defects at 2, 4, and 8 weeks after surgery (Fig. 2) demonstrated opaque calcified shadows bridging both ends of defects as early as 2 weeks in the group implanted with β-TCP combined with rhBMP-2 and polymer. Calcification became more evident at 4 weeks, and newly formed bone connecting both ends of defects appeared to have been remodeled into cortical bone with a bone marrow cavity. The BMP-loaded group showed a time-dependent increase in callus of nearly 100% at 6 weeks, but the group with β-TCP and polymer without rhBMP-2 showed only small amounts of newly formed bone formation—less than 20%. No bone formation was recognized in the control group (Fig. 3).
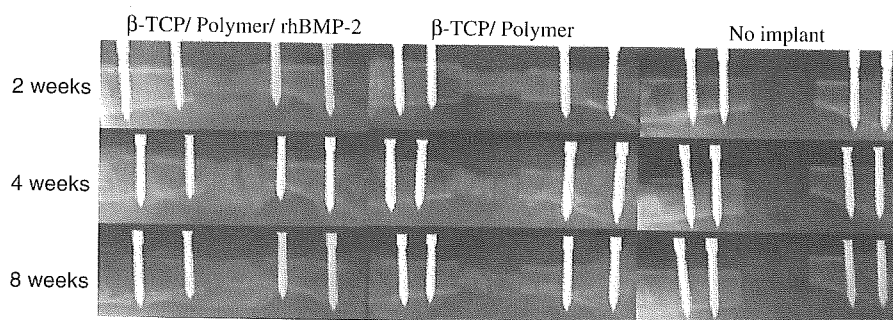
3.2. Mechanical and remodeling properties of the repaired bone by rhBMP-2/PLA-DX-PEG/β-TCP with long-term observation (Study 2)

Femurs in this group were stable and enabled free movement in caged animals until their sacrifice at 24 weeks. Radiology of femurs harvested 24 weeks after surgery showed complete regeneration of the intercalated defect. The 3D CT image and frontal tomographic image of regenerated femurs showed that normal femur anatomy had been restored with cortical bone with no residual evidence of implanted β-TCP cylinder blocks (Fig. 4).

3.2.1. Dual-energy X-ray absorptiometry analysis

Bone mineral density of femurs regenerated by biodegradable bone-inducing implants at 24 weeks was $350 \, mg/cm^2$, essentially equal to control (normal) levels. No significance was seen between experimental and control groups.

3.2.2. Biomechanical properties of repaired bone

The 3-point bending test of femurs regenerated by biodegradable bone-inducing implants at 8 weeks showed significantly lower stiffness (160 N/m) than
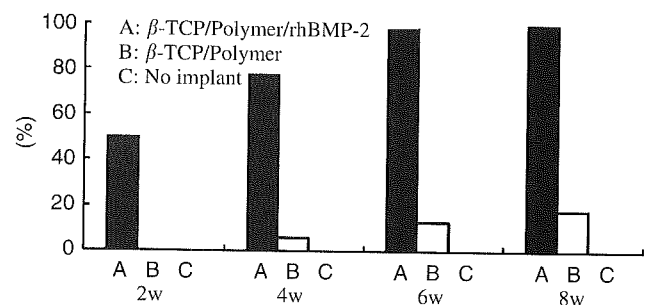
Fig. 3. BMP-loaded group promoting a time-dependent increase in callus (nearly 100% at 6 weeks). The group of β-TCP and polymer without rhBMP-2 promoted only negligible new bone formation—less than 20%. No bone formed in the control group.

Fig. 2. Representative femur radiographs. From left, implanted with β-TCP with PLA-DX-PEG and rhBMP-2, β-TCP with PLA-DX-PEG without rhBMP-2, and critical size bone defect without implantation (sham surgery). Sequential radiographs show bone repair at 2, 4, and 8 weeks after implantation in the experimental group.
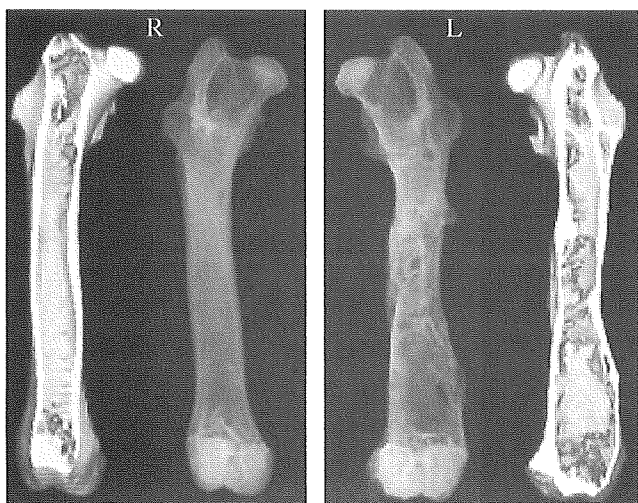
Fig. 4. Soft X-ray and 3D CT images of femurs 24 weeks after surgery. The repaired defect with biodegradable bone-inducing implants is shown at right (L). Images at left are of the counterpart femur in the control rabbit (R). The external fixator was removed 8 weeks after surgery. Note that β-TCP was absorbed and cortical walls remodeled anatomically with the marrow cavity.
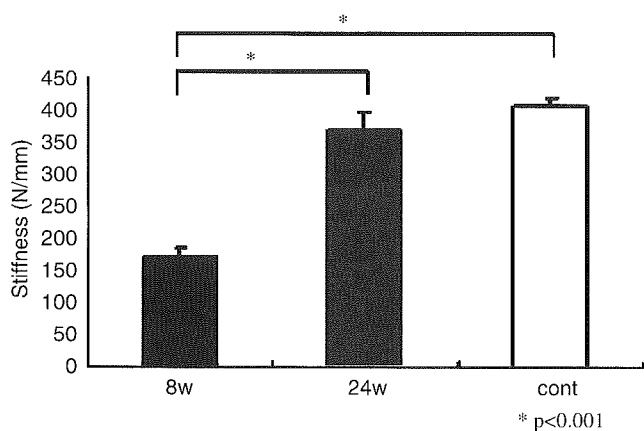
controls (400 N/m, nonsurgical femurs 24 weeks after surgery). Stiffness increased at 24 weeks (370 N/m) and was essentially equal to control (normal) levels (Fig. 5).

3.2.3. Histological findings

In histological sections from defect sites 2 weeks postoperatively, fibrous tissue and a thin layer of bone running parallel to the long axis and encasing implants were seen in the experimental group. A femur from the experimental group at 4 weeks with increased bone mass connected to β-TCP implants and fibrous tissue was observed. Regenerated cortical bone had united with ends of the original cortex of the femur. In sections from the experimental group 8 weeks postoperatively, bone occupying defects had remodeled to where cortical bone and hematopoietic marrow-like tissue were clearly visible (Fig. 6). On the β-TCP surface at 4 weeks, large numbers of TRAP-positive multinucleated cells (osteoclasts) appeared (Fig. 7). In sections of regenerated defects at 24 weeks, cortical bone was remodeled to lamellar bone connected to original ends of the femur. Marrow tissue was also completely restored and no remnants of β-TCP implants were visible (Fig. 8).

Fig. 5. Results of 3-point bending tests at 8 and 24 weeks after surgery (bar chart). (*: significant difference, $p < 0.001$).

4. Discussion

Our experimental results indicated successful regeneration of a critical intercalated bone defect in femur implantation using porous β-TCP with rhBMP-2 and a synthetic PLA-DX-PEG block copolymer as its delivery system. This approach clearly demonstrated that combining these synthetic materials and recombinant protein repaired large defects. The osteogenic potential of composite implants has yet to be compared critically, however.

The rhBMP-2 dose and rhBMP-2 concentration in polymer we used was determined based on previous study of critical bone defect repair in rabbits in which 1.5 cm of an intercalated bone defect in the humerus was
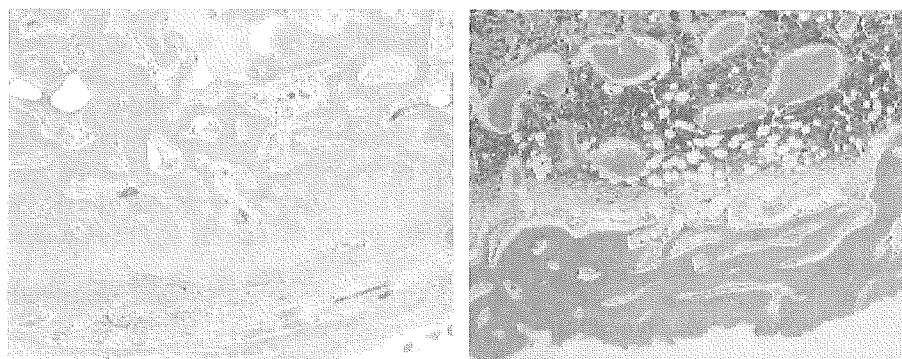

Fig. 6. Typical histological sections at 4 weeks (left, HE staining × 40) and 8 weeks (right, HE staining × 40). Abundant bone formed around β-TCP but not the outside of the femur in sections of specimens at 4 weeks. Cortical and bone marrow-like tissue clearly identified at 8 weeks.
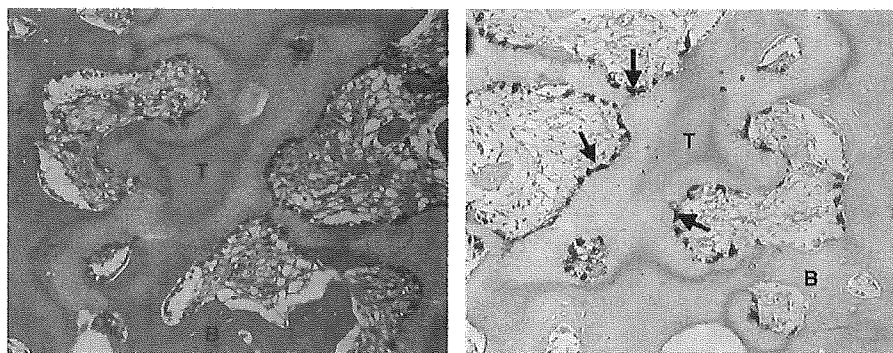
Fig. 7. Histological section of decalcified specimens harvested at 4 weeks and stained with HE (left, ×40) and tartrate-resistant acid phosphatase (TRAP) (right, ×40). β-TCP blocks coated with PLA-DX-PEG and rhBMP-2 are surrounded by abundant TRAP-positive multinucleated osteoclasts (arrows, stained red). (B = bone, T = β-TCP).
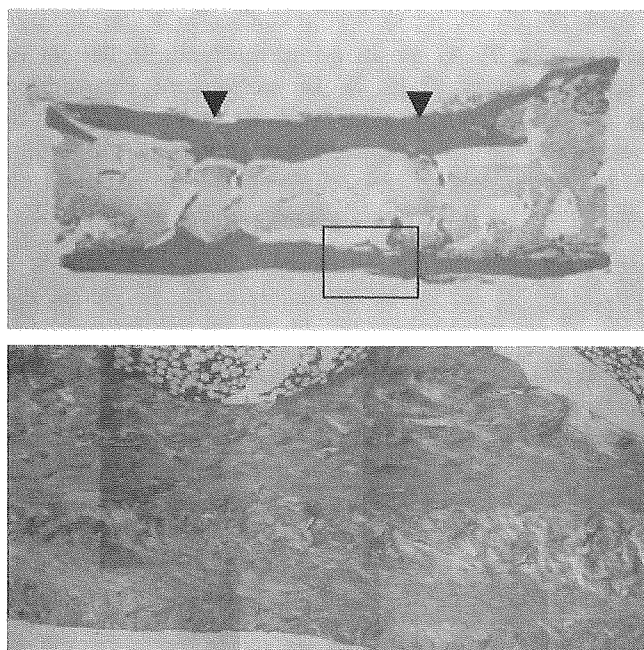

Fig. 8. Images of sagittal histological section at 24 weeks. Cortical walls were repaired completely. Arrowheads indicate fixator pin insertion locations. Polarized magnified image of junction between original and repaired bone (rectangle) (below). Randomly arrayed collagen in the repaired cortical wall contrasts with the more ordered layers of collagen in the original cortical wall.

successfully repaired by filling the defect with titanium mesh cylinders impregnated with a delivery system containing rhBMP-2 [17]. Titanium cylinders were not resorbed in repaired bone. Sustained permanent release of metal ions from the implant could increase the potential risk of allergic reactions or carcinogenesis, especially in infants. For these reasons, we replaced titanium in the present study with biodegradable material. As expected, β-TCP was completely resorbed and replaced by host bone within 24 weeks with no apparent adverse events from resorbed β-TCP.

The successful regeneration of the critical bone defect may be due in part to the porosity of β-TCP cylinders having 100–400 μm pores, which may enable rhBMP to accumulate locally within pores to where osteogenesis is initiated [22–25]. An appropriate local rhBMP concentration in pores would in turn enable successful invasion and ingrowth of mesenchymal cells in the implant and subsequent differentiation into osteoblasts. Additionally, the porosity of β-TCP appeared to be crucial to its rapid degradation in host animals, facilitating contact with host cells and resulting clearly in large numbers of osteoclasts contacting β-TCP [26]. The ability of these cells to permeate and resorb the β-TCP mass likely contributed to the relatively rapid replacement of implants by bone and marrow in our study. Although the location of BMP receptors on the surface of osteoclasts was reported previously [27], the effect of rhBMP-2 on osteoclastic differentiation remains to be clarified, requiring further study to determine the potential action of BMP on osteoclasts to explain the increased recruitment of osteoclasts in new bone induced by rhBMP2. Biodegradable osteoconductive β-TCP combined with a BMP delivery system is replaced by fully integrated biomechanically competent bone, eliminating one of the major limitations of other osteoconductive biomaterials.

The newly formed bone repairing the defect was remodeled to restore the normal anatomy of the original bone with concurrent resorption of β-TCP in 8 weeks. This means that BMP-induced new bone could remodel to adapt to the local biomechanical environment. Osteoconductive material must disappear to generate the physiological biomechanical environment and restore the original anatomy. Our results suggest that absorbable β-TCP is suitable as an ideal bone graft substitute.

The physicochemical properties and degradation profiles of polymer used in this study have been reported elsewhere [13–15]. A detailed safety check for clinical use of the polymer is currently in process. No systemic or local adverse effects have been noted to date.

The optimal content of rhBMP-2 in the carrier required to elicit new bone formation depends on the animal host [16–18], and a higher dose of rhBMP-2 is required in highly evolved animal species. Determining the optimal clinical dose thus requires additional experiments in primates.

5. Conclusion

A new absorbable bone graft substitute with osteogenic capacity was made by combining 3 artificial materials—porous β-TCP, rhBMP-2, and a PLA-DX-PEG delivery system. The capacity of this composite implant to regenerate bone is satisfactory. The composite implant was completely absorbed and replaced by newly formed bone, then remodeled into the femur to restore the natural anatomy. Although further safety checks and clinical trials are required, the practical use of this implant to promote bone regeneration is without doubt a realistic possibility.

Acknowledgements

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骨組織再生

Regeneration of the bone tissue

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Summary

Regeneration of the bone tissue has been required in various clinical scenes, especially in orthopaedic surgery, dental and oral surgeries. Regeneration of tissues requires cells, cytokines (growth factors) and scaffold. In the field of bone regeneration, bone morphogenetic proteins (BMPs) have utilized as main cytokines with osteoinductive capacity. We introduce the efficacy of implants composed of recombinant BMP and biodegradable polymers and β-tricalcium phosphate (β-TCP) as new scaffold or drug delivery system for the BMP to regenerate various types of bone defects with potential use in clinical practice as bone graft substitute.

Although various lines of researches for the bone regeneration have been progressed all over the world with variety of technologies including tissue engineering and gene therapy, these have problems remained to be solved and appear to be fur from practical use so far.

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背 景

　近年，さまざまな組織における再生医学技術が進歩し，一部が先端的医学として実際に臨床応用されつつある。骨組織再生は，必要な部位に必要な量・形態の骨を形成することを目的としている。臨床的には，脊椎疾患に対する脊椎固定術・外傷や腫瘍摘出に伴う巨大骨欠損の再建・顔面骨再建などで必要とされている。従来は骨再生の目的には自家骨移植が用いられてきた。しかし，自家骨移植による骨再生にはさまざまな問題があることが指摘されてきた。採取部の外科的侵襲や慢性的疼痛，知覚障害，変形などである。さらに移植骨の採取量の限界，力学的強度の不足など多くの問題があった。それらの欠点を補う方法として同種骨移植や生体親和性生体材料が用いられることもあったが，それらには骨形成促進（骨誘導）能力に欠けるという決

定的な問題点があること，また同種骨移植ではウイルス感染などの危険性や，保存，運搬などのシステム（骨銀行）が必要であるなどの理由で，わが国では汎用には至っていないのが現状である。したがって，臨床的な実用という面からみても，さらに優れた骨再生技術が求められている。理想的な骨再生技術または骨再生材料に求められる仕様としては，①移植材作成に生体への外科侵襲を伴わないこと，②生物由来でなく合成材料であること，③骨形成促進活性を有すること，④生体吸収性があり，骨再生完成後消失すること，⑤形成される骨の量・形態の制御が可能であること，⑥力学的強度に優れること，⑦可塑性があり，容易に成型できること，⑧生体に対する安全性に優れること，⑨安価で経済性に優れていること，⑩保存・搬送が容易であること，などが求められる。このような特性を満足する実用的な再生医療技術を可能にする材料を開発することは必ずしも容易ではない。

組織再生という生物現象には3要素が必須であるとされる。①分化能を有する未分化細胞，②細胞の増殖・分化を刺激する成長因子/サイトカイン，③その細胞が増殖分化する足場（Scaffold）である。骨組織の再生では，未分化間葉系細胞とそれらを軟骨細胞や骨芽細胞に分化誘導するサイトカインである一群の骨形成蛋白（Bone morphogenetic proteins：BMPs）が大きな役割を担っている。ScaffoldはBMPの担体であり，動物由来のコラーゲンが主に用いられてきた。

BMPsは，1965年にDr. Uristが，塩酸処理骨基質による異所性新生骨の誘導を報告し，その存在を提唱した[1]。その後，長期間にわたりさまざまな研究がなされたが，抽出は困難を極めた[2]。1980年代後半，アメリカGenetic Institute社により遺伝子情報がcloningされ，リコンビナント蛋白として分離・精製された[3]。現在では，骨再生医療の基本的な材料としてヒト型リコンビナントBMP（Recombinant human：rhBMP）が用いられている。すでに限られた用途（脊椎前方固定）で臨床利用が始まっている[4]。しかし，使用については多くの問題があり，その解決が迫られている。

本稿ではBMPを用いた骨再生技術の現状について記述する。

BMPの生物化学的特性

BMPsは，正常骨芽細胞系細胞によって産生分泌されるホモ2量体の活性蛋白（サイトカイン）である。その成熟領域の分子構造の特徴として，分子量は30kDa前後で疎水性の強い中性蛋白分子である。また，C末端からみたアミノ酸配列では7個のシステイン残基を有し，その位置がTransforming growth factor-β（TGF-β）と同一である。したがって，BMPsはTGF-β super-familyに属する一群のfamilyをなしている（図1）[5]。

現在ではBMP-2～BMP-15の14種類の分子が同定されている。このうち骨形成促進活性を有して異所性に骨形成を誘導する活性が確認されているのはBMP-2，BMP-4，BMP-6，BMP-7（OP-1），BMP-9である[6]。それぞれのBMPの詳細な生理学的役割の違いは明らかではないが，発生過程[7]や骨折治癒過程[8]でみられる骨形成にはBMP発現が必須である。

BMPの分子構造にはcystine knotと称する安定化構造があり，極めて安定した蛋白である。したがって，変性しにくく，90℃30分程度の熱処理でも生物活性は保たれる。すなわち，保存・搬送に関する問題はほとんどないとされる。

BMPによる新生骨誘導

rhBMPの生体内埋植による局所的骨形成促進効果は，これまで多くの実験で示されている。大きな骨欠損修復や脊椎固定がrhBMPによって可能であることも実験動物での結果が報告されている[9]。実際の臨床治療試験もすでに一部行われており，欧米では脊椎固定，骨折後偽関節治療などでの限定的使用が許可されている[10]。しかし，BMPの臨床応用のためには，まだ改良を加え解決しなければならないいくつかの問題が残されている。現時点での主な問題点は，BMPの担体としてウシのコラーゲンが使用されていることからくる狂牛病などのリスク，ヒトでのBMPに対する低い応答性に由来する起因した高用量のBMPの必要性，その結果として高価となっており一般

的普及が困難な点である。この問題解決には新しい安全な担体の開発，低用量BMPで骨再生を可能とする新しい担体（drug delivery system：DDS）の開発が望まれている。リコンビナントBMPとして実際に臨床応用されているBMPは，BMP-2およびBMP-7（OP-1）であるが，いずれについてもこのような欠点が解決されていない。

脊椎固定術に関して欧米では，リコンビナントBMPと担体（コラーゲン）を一体化して生体材料として用いている。rhBMP-2についてはMedotronic Sofamor Danec社から，またBMP-7（OP-1）についてはStryker社から発売されている。わが国でのBMPの臨床応用はいまだ認可されていない。

BMPの効果的担体

重要な問題はBMPをいかに優れたDDSによって生体内の必要な限局した部位に作用させ，より安全かつ効果的に骨形成を促進するかである。現在使われている担体であるコラーゲンの使用についてはBSEなどの感染症問題が指摘されており，さらに力学的強度に欠ける点などの問題があり，今後コラーゲンに代替し得る担体の開発は骨組織の再生医療を拡大していく上で急を要する。すなわち，BMPの骨形成促進活性を有効に利用するためには，安全で安価な生体吸収性で成型しやすい合成担体が必要である。我々はこのような特性を有する担体として数種類の合成ポリマーを開発した（poly-


図1 TGF-βスーパーファミリー
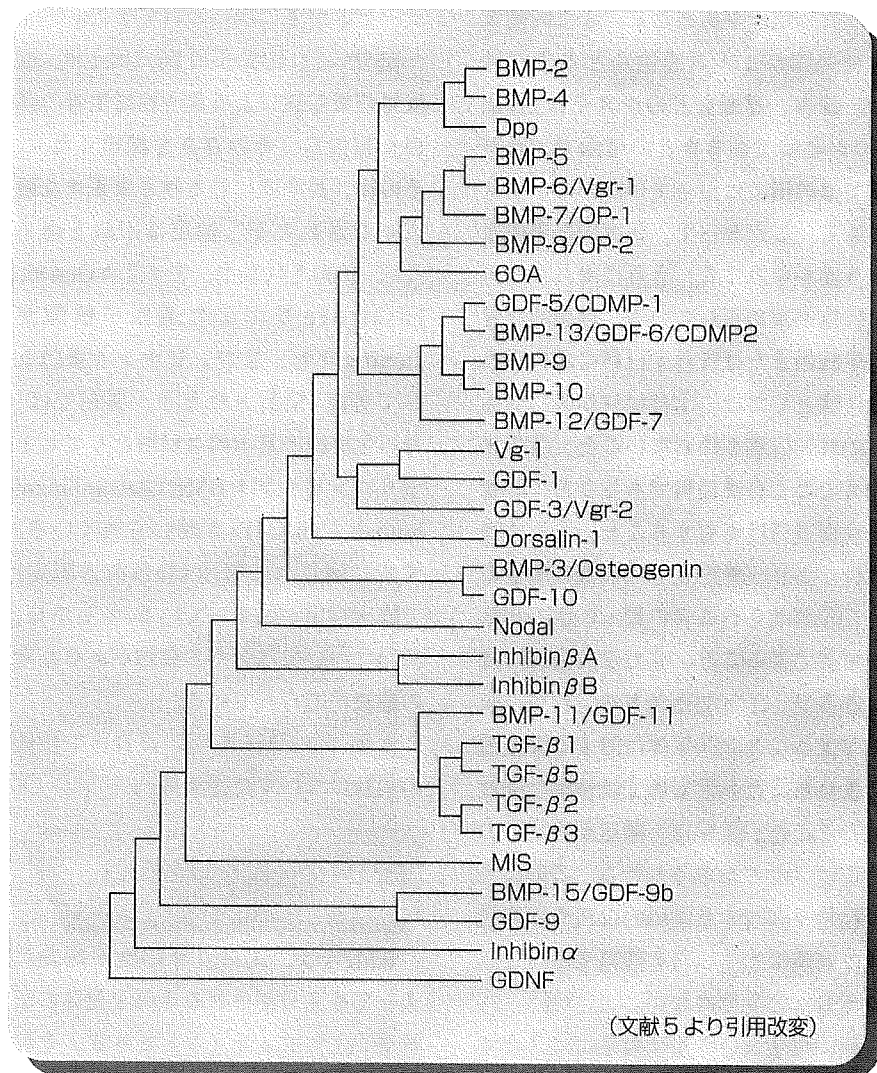
（文献5より引用改変）

lactic acid-polyethylenglycol block co-polymer：PLA-PEG）[11]。このpolymerに少量のBMPを混和して生体に埋植すると異所性にも同所性にも骨新生が起こり，埋植したpolymerと置き換わる。このpolymerの詳細についてはすでに報告してあるので，ここでは省略する。このBMP/polymer複合材料を用いると比較的大きな骨欠損を修復で

きる。図2はラットの頭蓋骨欠損の例を示す。しかしこのpolymerの欠点として，常温では粘着性が強いために扱い難い点である。この点を改良するために，生体吸収性β-tricalcium phos-phate（β-TCP）粉末をpolymerと等量混和することで粘土状にした。これによってインプラント材料の容積がほぼ倍加し，形成される骨量もほぼ倍加す
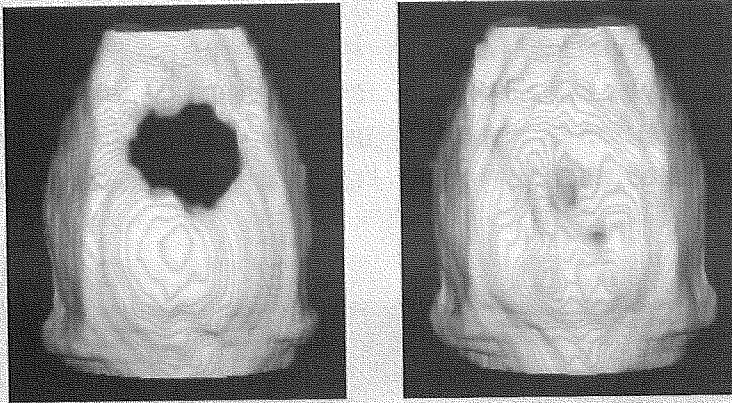
BMP非含有(ポリマー単独)群では頭蓋骨は欠損
したまま(左図)であるが,BMP含有ポリマー移
植群(rhBMP-2/5μg・ポリマー/30mg)では,
移植後6週間で骨欠損部はほとんど認めず骨組織
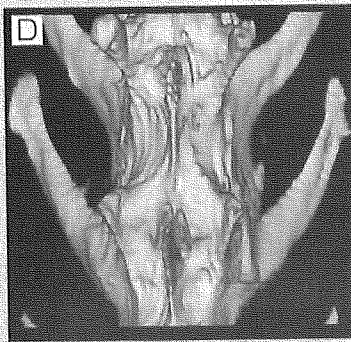が再生されている。 (文献23より引用改変)

図2　ラット頭蓋骨欠損モデル3D-CT画像
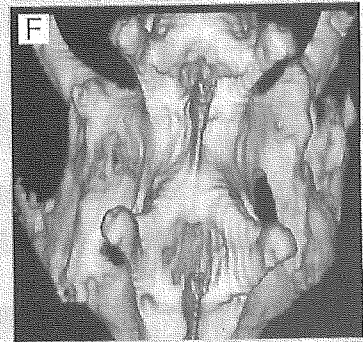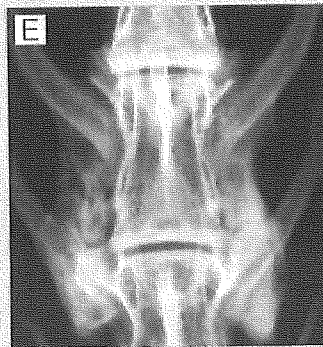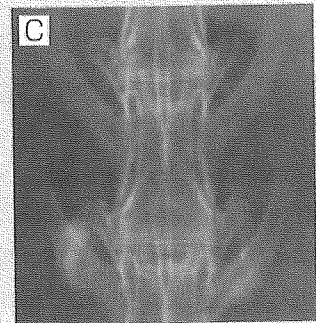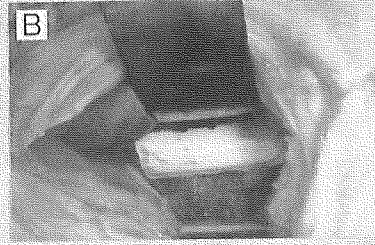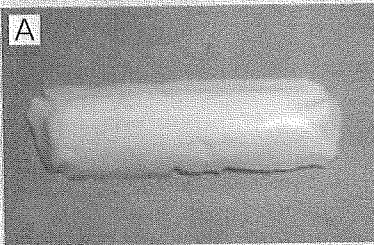
A：rhBMP-2・β-TCP・生体吸収性ポリマーで作成したImplant Composite
B：埋殖風景
Implant埋殖後6週の軟X線像(C, E)および3D-CT画像(D, F)。BMP非含有群(C, D)では横突起間に骨形成を認めないが,
rhBMP-2(15μg)含有群(E, F)では旺盛な骨形成を認める。力学試験においても有意な固定強度が得られた。
(文献12より引用改変)

図3　rhBMP-2・β-TCP・生体吸収性ポリマーによる日本白色家兎脊椎固定(➡ 巻頭Color Gravure参照)

るため結果としてBMPの骨形成効率を上げることも可能となる。また粘着性を減ずることで扱いやすい材料にできる。このBMP/polymer/β-TCP powder複合材料を用いた動物での脊椎後側方固定を図3に示す[12]。

多孔性生体材料の孔内にBMP/polymerを封入することで、立体的構造をもったインプラントを構成し骨欠損再生も可能である。ウサギ大腿骨の1.5cmのcritical defectの再生例を図4に示す[9]。この例のように多孔性β-TCPを用いれば生体材料を残さずに骨再生が可能である。この材料を使えばイヌの8cmに及ぶ欠損の再生も比較的短時間で可能である（図5）。

BMPに対する反応性の種差

rhBMPを普遍的に臨床応用するための大きな障害として、ヒトでのBMPに対する応答性が低いことがある。その結果として高用量のBMPを必要とし、必然的に高価な治療法とな

ることがあげられる。コラーゲンを担体とした場合、ヒトでは1cm³の新生骨を形成するためには約1mgのBMPが必要である。上述の新規担体では約半分の用量にできる。できるだけ低用量のBMPで効果的に骨再生を

達成するための工夫も必要である。そのための工夫として①DDSを至適化すること、②BMPの生物活性を増幅する方法の開発、があげられる。またBMPの生物活性を増幅する方法として、我々はprostagrandin E₂（PGE₂）の

A：欠損後8週、骨欠損部に変化を認めない。
B：欠損部に生体吸収性ポリマー・β-TCP移植後8週、骨形成を認めない。
C：欠損部にrhBMP-2・生体吸収性ポリマー・β-TCP移植後8週、良好な骨形成を認め、完全に骨欠損は修復されている。
D：同24週（右）、非処理骨（左）とほぼ同等の径の骨修復を認める。

（文献9より引用改変）
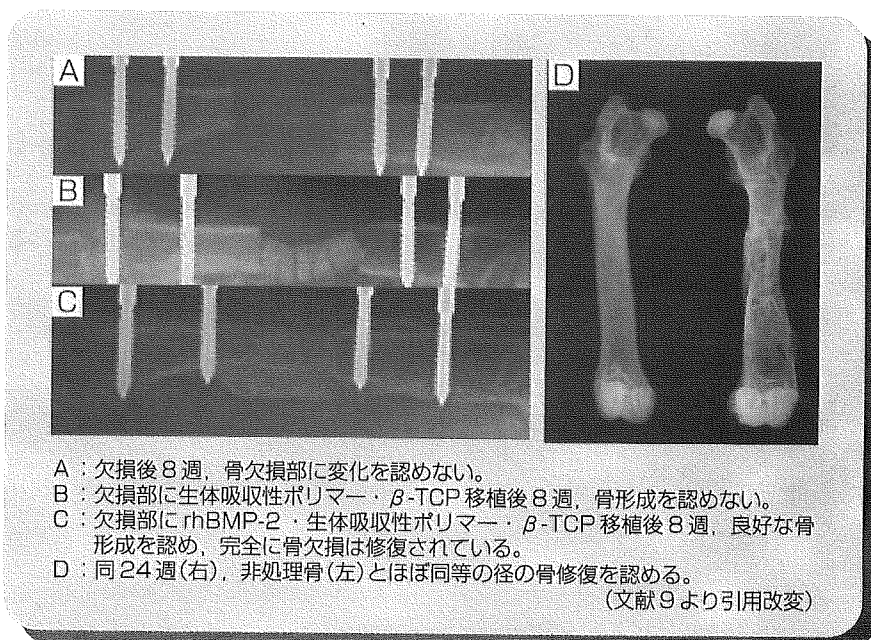
図4 日本白色家兎大腿骨巨大骨欠損モデルにおけるrhBMP-2・β-TCP・生体吸収性ポリマーによる骨欠損修復

Control群では骨新生は認められないが、BMP/polymer/β-TCP Composite移植群では、移植後3週ですでに旺盛な骨形成を認め、6～12週にかけて肋骨はリモデリングしており、さらにβ-TCPの吸収も認める。

図5 肋骨の再生（イヌ）

受容体EP4アゴニスト（ONO4819）[13]やヘパリンがBMPの生物活性を増幅する作用があることを明らかにしている。すなわち，polymerにBMPを加えてこれらを添加するとBMPの活性が約2倍強められることを見つけている[14]。より強力な増幅物質が開発できればさらにBMP用量を減少できる可能性がある。最近の研究報告ではSulfated polysaccharides[15]や人工的に作成されたある種のペプチドであるB2A2[16]がBMPの効果を増強させるなど多くの研究が進んでおり，in vivoでの効果増強の確認が期待される。将来的にこれらの薬剤を臨床応用することにより，より効果的・効率的な骨組織の再生が可能になると考えられる。

その他の骨再生医療

　BMP以外のサイトカインや増殖因子で，骨再生促進を図る研究も進みつつある。Kawaguchiらは，塩基性線維芽細胞増殖因子（basic fibroblast growth factor：bFGF, FGF-2)により，担体としてハイドロゲルを用いて，Non Human Primatesにおける骨折治癒を促進したと報告している[17]。また，bFGFによる骨折治癒促進ならびに歯槽骨欠損の再生は現在臨床試験が進行中とのことである。

　我々は骨再生医療における担体として，生体吸収性ポリマーおよびβ-TCPを主に用いて良好な実験結果を得ているが，その他にも数多くの担体が報告されている。α-TCPを主成分

としたリン酸カルシウム骨セメント[18]や連通多孔体を有したハイドロキシアパタイト[19]やハイドロキシアパタイト・ポリ乳酸複合多孔体[20]，多孔質ハイドロキシアパタイト・コラーゲン複合体[21]など種々の担体を用いた骨再生医療が試みられている。それらの有効性についての比較も今後の問題点である。

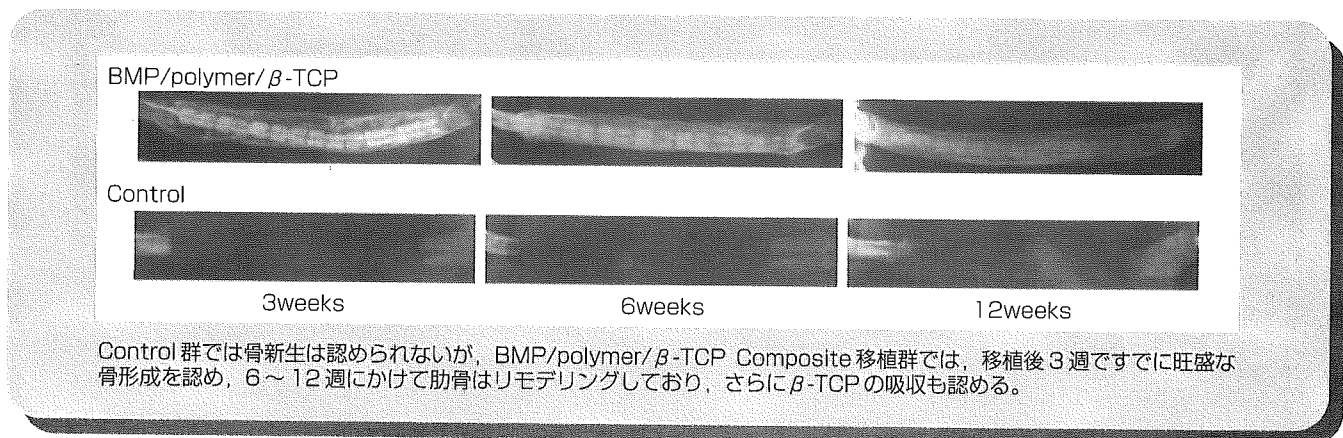　ここで述べた遺伝子組み換え体サイトカイン（蛋白）を使った骨再生法以外に培養系を用いた組織工学による方法がある[22]。未分化間葉系細胞または骨髄間質細胞を患者から採取して，生体外で培養し分化・増殖させる。次に，その細胞を多孔性生体材料の孔内に封入するなどして，担体（scaffold）と分化・増殖させた細胞とを患者の骨形成が必要な部位に移植して骨組織再生により治療する方法である。この際，採取される細胞の数に制限があり，短期間で骨芽細胞に細胞を分化増殖させなければならない。このためにBMPをはじめとした骨分化誘導物質を用いる。つまり，培養中にBMPやデキサメタゾン，β-グリセロリン酸，アスコルビン酸などを作用させて，未分化な細胞を選択的に骨形成細胞に分化・増殖させ効率良く目的の細胞を大量に得る方法であり，優れた培養技術と優れた生体材料を組み合わせて再生を行う試みである。実験的段階ではあるものの，近年数十例に対して臨床的に応用されつつあると報告されている。しかし，培養に伴う安全性確保，経費，長い所要時間などを考慮すると，実用

にはクリアすべき問題が多く，一般的な実用化には大きな困難が予想される。

　また，遺伝子導入による方法では遺伝子産物である蛋白を生体内に投与するのではなく，遺伝子そのものを細胞内に導入して蛋白を局所で合成させる方法である[6]。具体的には，種々のBMP遺伝子を組み込んだウイルスを骨形成が必要な部位の細胞に感染させて，局所的にBMPの産生を高める方法，あるいは患者の細胞を採取して生体外でBMP遺伝子を組み込んでBMP産生細胞とし，増殖させて骨形成が必要な部位へ移植して治療する方法などが考えられている。しかし，この遺伝子治療の研究はまだ実用には至っていない。まずは安全な発現vectorの開発ができていないこと，より効率的かつ低侵襲な遺伝子導入方法の問題，さらに骨組織再生が必要な限局した部位における遺伝子発現制御方法などさまざまな新技術の追加が必要になる。今後の大きな研究テーマの一つであろうが，実用化にはまだ距離があるように思われる。

最後に

　患者の高齢化，疾患および治療法の多様化に伴い，整形外科領域を中心に，有効な骨組織の再生医療の発展が急務となっている。本稿では，我々の研究結果を中心に骨再生医療について述べた。さまざまな骨再生を目指した基礎的研究が進行中であるが，遺伝子組み

換え体サイトカインであるリコンビナント BMP を用いた骨再生医療は実用化の面からはより先行した技術であると考えている。近い将来には，普遍的な骨組織再生医療技術として活用されると予想している。

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Augmentation of bone morphogenetic protein-induced bone mass by local delivery of a prostaglandin E EP4 receptor agonist

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Abstract

Recombinant human bone morphogenetic protein (rhBMP) is viewed as a therapeutic cytokine because of its ability to induce bone. However, the high doses of rhBMP required for bone induction in humans remain a major hurdle for the therapeutic application of this protein. The development of a methodology that would effectively overcome the weak responsiveness to human BMP is highly desired. In the present study, we investigate the ability of a prostaglandin E EP4 receptor selective agonist (EP4A) to augment the bone-inducing ability of BMP in a biodegradable delivery system. A block copolymer composed of poly-D,L-lactic acid with random insertion of p-dioxanone and polyethylene glycol (PLA–DX–PEG, polymer) was used as the delivery system. Polymer discs containing rhBMP-2 and EP4A were implanted into the left dorsal muscle pouch of mice to examine the dose-dependent effects of EP4A. Fifty mice were divided into 5 groups based on the contents of rhBMP and EP4 in the polymer (group 1; BMP 5 μg EP4A 0 μg, group 2; BMP 5 μg EP4 3 μg, group 3; BMP 5 μg EP4 30 μg, group 4; BMP 5 μg EP4 300 μg, group 5; BMP 0 μg EP4 30 μg, $n = 10$ each). All implants were harvested, examined radiologically, and processed for histological analysis 3 weeks after surgery. On dual-energy X-ray absorptiometry (DXA) analysis, the bone mineral content (BMC) of the ossicles was 6.52 ± 0.80 (mg), 9.36 ± 1.89, 14.21 ± 1.27, and 18.75 ± 2.31 in groups 1, 2, 3, and 4 respectively. In terms of BMC, the values of groups 3 and 4 were significantly higher than those of group 1. The mean BMC value of group 4 was approximately 3 times higher than that of group 1. No significant difference in body weight was noted among the groups during the experimental period. In summary, the presence of a prostaglandin E EP4 receptor selective agonist in the carrier polymer enhanced the bone-inducing capacity of rhBMP-2 with no apparent systemic adverse effects.
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Keywords: Bone morphogenetic proteins; Bone metabolism; Bone volume; Bone mineral density; Biomaterials

Introduction

Bone has an inherent regenerating potential, and damaged bone or fractures are repaired by local new bone (callus) formation in a period of several weeks after an injury. The regenerating potential of bone has been attributed to factors or molecules with the biological capacity to induce mesenchymal cells to differentiate into bone- or cartilage-forming cells (osteoblasts and chondrocytes) and thereby form the callus. Bone morphogenetic proteins (BMPs) were originally isolated on the basis of their ability to induce

ectopic cartilage and bone formation via an endochondral cascade when implanted in experimental animals [1]. 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 a clinical setting [2]. However, 2 problems need to be addressed before we can witness the widespread clinical use of rhBMPs. One issue involves the use of a carrier material that has adequate safety and efficacy for BMP delivery. Currently, bovine collagen is used clinically as a carrier for rhBMPs, but use of this material comes with the risk of contracting bovine spongiform encephalopathy (BSE) or Creutzfeldt–Jacob disease (CJD). These diseases are

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potentially transmitted by prion proteins through cattle-derived foods and implant materials. Another problem is the high dose of rhBMP required for clinical efficacy in human patients. For example, to achieve a single level of spinal fusion, several to 10 mg of rhBMP are required. This results in the high cost and limited use of BMP as a substitute for bone autograft. Large doses of BMP may also increase the risk of potential adverse events in patients [3–6].

To address the issue of finding a suitable carrier, we have developed new biodegradable synthetic polymers that work effectively to deliver rhBMP and elicit new bone formation consistently at the implanted sites. The combination of rhBMP-2 and the polymers has enabled the successful regeneration of critical-size bone defects in experimental animals [7–10].

To improve the performance of rhBMP, we have sought agents to reinforce the bone-inducing activity of the protein and increase the induced bone mass. To this end, we have examined phosphodiesterase (PDE) inhibitors (pentoxifylline, rolipram) and a compound (ONO-4819), which is a prostaglandin (PG) EP4 receptor selective agonist (EP4A) [11–13]. PGE produced by cells of the osteoblastic lineage has been implicated as a regulator of bone metabolism through stimulation of either bone formation or resorption [14–16]. Exogenously applied PGE, either systemically or locally, also has enhanced bone formation in in vivo experimental models [17–19]. These biological effects of PGE are mediated through PGE receptors, which have been classified into 4 sub-types, EP1 through EP4. These EP receptors are encoded by distinct genes and are expressed in a tissue-specific manner [20–25]. In general, PGE mediated via EP1 increases intracellular Ca^{2+} concentration, EP2 and EP4 increase cAMP, and EP3 reduces cAMP and modulates down-stream signaling [25]. Knockout mouse studies have revealed that EP4 is the major receptor that mediates the PGE_2-induced anabolic action in bone [26–30]. Systemic administration of an EP4 agonist (ONO-4819) enhanced new bone formation in mice, and an EP4 antagonist suppressed the increase in trabecular bone volume induced by PGE_2 [13,30–33]. In our previous study, the systemic administration of these drugs by daily injection for 1 week during the initial phase of BMP-induced bone formation led to a significant augmentation of ossicle mass [13]. These results suggest that the efficient local release of these activators for BMPs could induce augmented bone formation without adverse effects due to high dose and long-term administration. Therefore, we examined the effects of adding a low dose of ONO-4819 to the BMP delivery system on new bone formation.

Materials and methods

Drugs/chemicals/materials

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

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.

Poly-D,L-lactic acid–p-dioxanone–polyethylene glycol block copolymer (PLA–DX–PEG) (MW; 9800, PLA/DX/PEG molar ratio; LA/Dx/E0 = 43/14/43) was synthesized and provided to us by Taki Chemicals Co. (Kakogawa, Japan). The structural formula of the polymer is shown in Fig. 1. The polymer has a sticky gel-like character at room temperature and turns into a soft gel at 50°C. The physicochemical characteristics and the efficacy of this polymer as a carrier material for rhBMP-2 have been described by our group in previous reports [9,10]. The minimal optimal content of rhBMP-2 required to induce new bone formation was approximately 1 μg in 20 mg of the polymer (0.005%) in mice, 0.02% in rabbits, and 0.04% in dogs based on our previous experimental data [8,10,34].

Animals

One hundred and ten closed colony male ICR mice (4-weeks old; Nippon SLC, Hamamatsu, Japan) 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.

Preparation of PLA–DX–PEG polymer implants containing rhBMP-2 and ONO-4819

To prepare a single implant, 30 mg of the PLA–DX–PEG polymer was softened by heating to 37°C, mixed with an aliquot of either the rhBMP-2 solution (0.5 μg/5
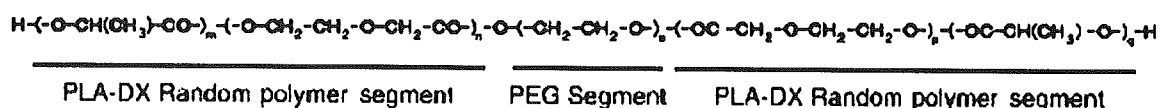
H-(-O-CH(CH₃)-CO-)ₘ-(-O-CH₂-CH₂-O-CH₂-CO-)ₙ-O-(-CH₂-CH₂-O-)ₒ-(-OC -CH₂-O-CH₂-CH₂-O-)ₚ-(-OC-CH(CH₃) -O-)�q-H

| PLA-DX Random polymer segment | PEG Segment | PLA-DX Random polymer segment |

Fig. 1. Structural formula of PLA–DX–PEG polymer. Structural formula of the poly-D, L-lactic acid with random insertion of p-dioxanone and polyethylene glycol block copolymer (PLA–DX–PEG). The subscripts m, n, o, p, and q represent variable numbers of these units.

μl or 5 μg/5 μl) or rhBMP-2 and ONO-4819 solution (3 μg/3 μl, 30 μg/3 μl, 300 μg/3 μl) and then fabricated into a disc (6 mm diameter, Fig. 2). In summary, 0, 3, 30, or 300 μg of ONO-4819 was mixed with the polymer plus 5 μg of rhBMP-2 and implanted into mice in each group (5 mice in each group and 1 implant/mouse). To examine the effects of ONO-4819 alone, 30 μg/8 μl was added to the polymer without rhBMP-2. All procedures were carried out under sterile conditions. The implants were stored at −40°C in a freezer until required for implantation.

Experimental design

To examine the dose-dependent effects of the EP4 receptor agonist on ectopically induced bone formation by rhBMP-2, 50 mice were divided into 5 groups (10 mice per group). The mice were anesthetized by diethyl-ether gas inhalation, and the PLA–DX–PEG polymer discs prepared as described above were surgically implanted into the left dorsal muscle pouches (one pellet per animal) of the mice. In group 5, polymer discs containing 30 μg of ONO-4819, but no rhBMP-2, were implanted in the same manner.

1. 5 μg of rhBMP-2 per animal
2. 5 μg of rhBMP-2 and 3 μg ONO-4819 per animal
3. 5 μg of rhBMP-2 and 30 μg of ONO-4819 per animal
4. 5 μg of rhBMP-2 and 300 μg of ONO-4819 per animal
5. 30 μg of ONO-4819 per animal

At 1, 2, and 3 weeks after surgery, the body weight of each mouse was measured and recorded. Three weeks after surgery, the mice were sacrificed, and the implants were harvested and processed for histological analysis following morphological and radiological examination.

Radiological and histological analyses for rhBMP-2 induced ectopic bone

All harvested tissues were radiographed with a soft X-ray apparatus (Sofron Co., Ltd., Tokyo, Japan). 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 (DCS-600EX, Aloka Co., Tokyo). The ossicles or tissue mass from each group was then fixed in neutralized 10% formalin, decalcified with K-CX (Fujisawa Pharmaceutical Co., Ltd. Japan), dehydrated in gradient ethanol series, and embedded in paraffin wax. Sections of 3 μm thickness were cut, stained with hematoxylin–eosin, and observed under a light microscope.

Bone metabolic markers in mice

To investigate the anabolic effects of ONO-4819 on systemic bone metabolism, an additional 60 mice were divided into 3 groups as follows: sham-operated mice that received sham operation and lacking implants (10 mice per group), group 1: 5 μg of rhBMP-2 per animal (5 mice per group) and group 3; 5 μg of rhBMP-2 and 30 μg of ONO-4819 per animal (5 mice per group). Blood samples were collected from mice of each group at 1, 2, and 3 weeks. The samples were stored at −80°C until biochemical analysis. Serum osteocalcin was measured by immunoradiometric assay (IRMA) using a commercial kit (Immutopics, Inc. San Clemente, CA) according to the manufacturer's instructions. Total alkaline phosphatase (ALP) activity, calcium (Ca), and phosphate (P) in serum were also measured in each group with commercially available kits.

Statistical analysis

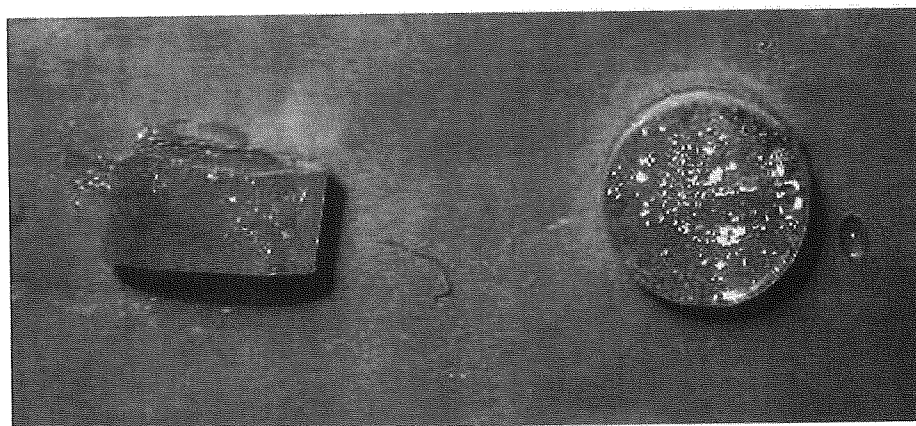Data are presented as mean ± SE. The degree of significance was determined by post hoc testing using the

Fig. 2. PLA–DX–PEG polymer disc. Photograph of 6-mm-diameter PLA–DX–PEG polymer disc. The polymer has a hard sticky gel-like property at room temperature and softens when heated to 50°C.

Bonferroni method. An associated probability (*P* value) of <0.05 was considered significant.

Results

Body weight changes in animals

In our previous experiments, mice that received systemic injection of an excessive dose (100 μg/kg) of ONO-4819 every 8 h for 3 weeks showed a significant decline in body weight gain. In the current experiments, no significant difference in body weight gain was noted among the groups that received implants with or without local release of ONO-4819 (Fig. 3).

Radiological and histological evaluations

Pieces of hard tissue were harvested from the implantation sites of mice from groups 1, 2, 3, and 4 at 3 weeks after implantation. In group 5 (ONO-4819, 30 μg without BMP-2), no evidence of hard tissue formation was found at the implantation sites. On soft X-ray radiograms, the calcified samples retrieved from the mice revealed a trabecular network encased within a shell-shaped bone layer (Fig. 4). Histological sections of these samples showed normal characteristics of bone with trabeculae and hematopoietic marrow in the inter-trabecular space, findings that were also common to ossicles from groups 1, 2, 3, and 4. (Fig. 5) Radiological images indicated that the ossicles from group 3 (rhBMP-2, 5 μg + ONO-4819, 30 μg) and 4 (rhBMP-2, 5 μg + ONO-4819, 300 μg) were larger than those observed from control group 1 (rhBMP-2, 5 μg without ONO-4819).

On DXA analysis, the bone mineral content (BMC) of the ossicles containing ONO-4819 increased in a dose-dependent manner (3, 30, and 300 μg groups were 9.36 ± 1.89 mg, 14.21 ± 1.27 mg, and 18.75 ± 2.31 mg, respectively) Ossicles from group 1 mice (without ONO-4819) had a BMC of 6.52 ± 0.80 mg. In terms of BMC, the values of groups 3 and 4 were significantly higher than those of group 1. The mean BMC value of group 4 (BMP-2, 5 μg + ONO-4819, 300 μg) ossicles was approximately 3 times higher than that of the control group (Fig. 6).
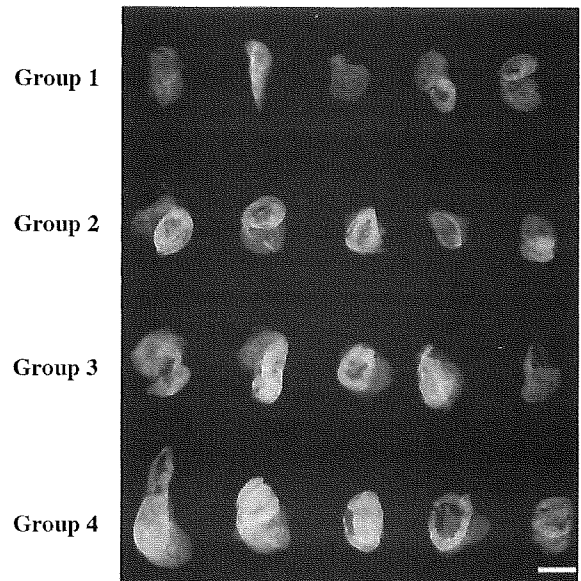
Fig. 4. Radiographic findings. Soft X-ray photograph of the ossicles harvested at 3 weeks after implantation (bar = 5 mm). A typical implant from each group is shown (groups 1, 2, 3, and 4). Both the radio-opaque areas and radiological densities of the ossicles on the radiogram were larger in groups 3 and 4 than in control group 1.

Serum osteocalcin and ALP activity assay

At 1 week, both serum osteocalcin (299.8 ± 24.4 ng/ml) and ALP activity (495.2 ± 32.0 IU/l) levels significantly increased in group 3 compared to the sham-operated animals (osteocalcin 208.6 ± 25.6 ng/ml, ALP activity 356.0 ± 39.8 IU/l). At 2 weeks, serum ALP activity (439.0 ± 76.8 IU/l) levels had increased significantly when compared to the sham-operated animals (ALP activity 313.2 ± 12.1 IU/l) (Fig. 7A). However, there were no significant differences among the groups at 3 weeks after implantation (Fig. 7B). In addition, there was no significant increase in serum calcium and phosphate level among them at any time point (data not shown). No significant changes in serum osteocalcin and ALP levels from the baseline were recorded in the groups that received implants containing ONO-4819.

Discussion

Based on these data, EP4A was examined for its ability to enhance BMP-induced bone formation and improve rhBMP-2 performance. In our previous study, systemic subcutaneous injections of the EP4A (ONO-4819) for 3 weeks increased bone mass induced by rhBMP-2 and caused a decline in body weight gain in the experimental animals [13]. To achieve the anabolic action and avoid the systemic adverse effect, low doses of the drug were added to the degradable polymer carrying the rhBMP-2 and implanted into the host mice. In this study, in a very encouraging response, ONO-4819 significantly increased the BMP-induced bone mass in dose-dependent manner
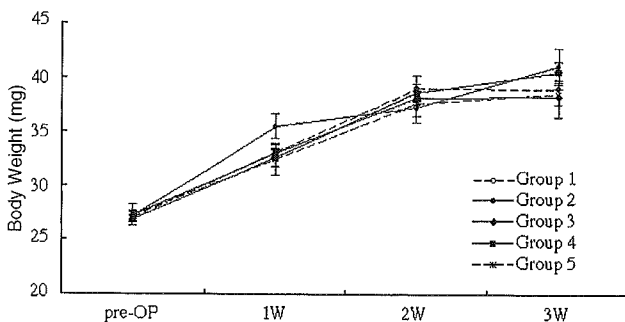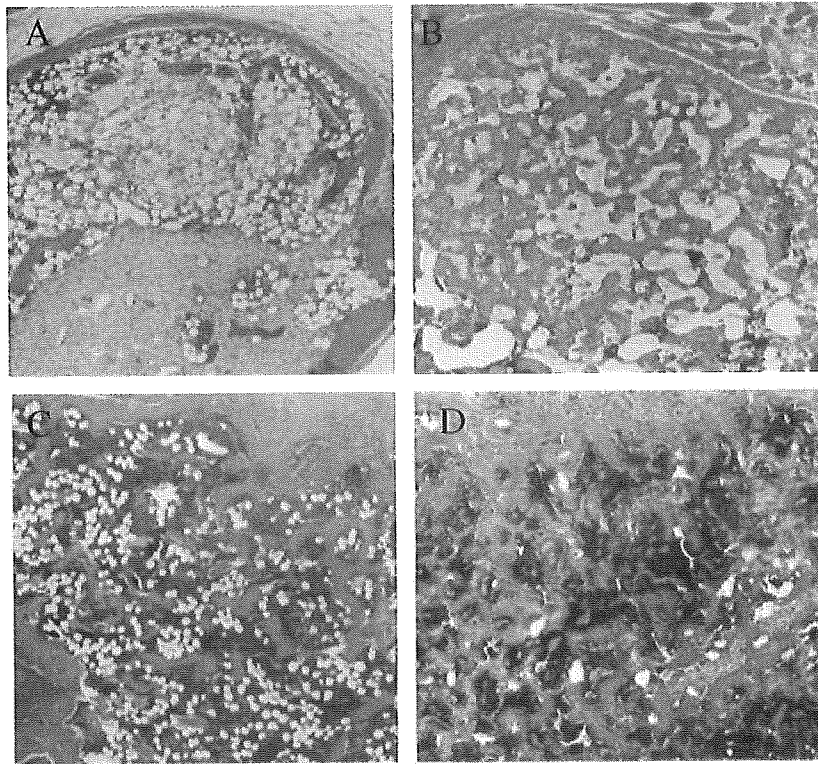

Fig. 3. Body weight. No significant difference in body weight was noted among the groups with implants with or without ONO-4819.

Fig. 5. Histology. Histological sections of the ossicles at 3 weeks after implantation are shown (hematoxylin–eosin stain; original magnification ×40). (A) group 1: 5 μg of rhBMP-2, (B) group 2: 5 μg of rhBMP-2 and 3 μg of ONO-4819, (C) group 3: 5 μg of rhBMP-2 and 30 μg of ONO-4819, (D) group 4: 5 μg of rhBMP-2 and 300 μg of ONO-4819. New bone formation with hematopoietic marrow and bony trabeculae was visible in the rhBMP-2-induced ossicles. In groups 3 and 4, there were visible increases in the number and thickness of bony trabeculae when compared to the ossicles from group 1.

without significant body weight loss. The total dose of ONO-4819 required for a doubling of the BMP-induced bone mass was reduced when compared to the dose required using consecutive systemic administration (3 injections/day for 3 weeks) of the drug.

Enhanced bone formation by systemic administration of the EP4A over an experimental period of 3 weeks was essentially reproduced by the local release of the agent over the first week following implantation. This is the period when young mesenchymal cells most likely migrate, proliferate, and infiltrate the BMP/polymer composite implants before new bone formation gets underway
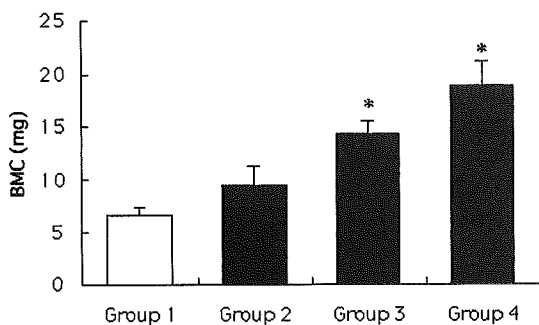

Fig. 6. Bone mineral content. The bone mineral content (BMC) of the ossicles at 3 weeks after implantation. BMC of ossicles was dose-dependently higher in groups 2, 3, and 4 than those in the group 1. Data expressed as mean ± SE. *Significantly different from controls ($P < 0.05$).

[7,9,10]. It is possible that these young mesenchymal cells were responsible for the bone formation enhanced by EP4A. Therefore, a low dose of the EP4A, ONO-4819, delivered locally and concurrently with rhBMP enhanced new bone formation and significantly increased bone mass. The effective period of local release of the EP4A is not greater than 2 weeks based on the degradation rate of the polymer [9,10]. Therefore, one possible explanation for the bone mass increased by EP4A is that EP4A works first in osteoblast precursors with a potential for chondro-osseous differentiation in the early phase of the bone-forming reaction. In the previous study, due to identifying the time phase when ONO-4819 exerts its pharmacological effects, EP4A was systemically administered for 1 week over pre (−1–0 week), initial (0–1 week), middle (1–2 week), or late (2~3 week) phase, respectively. The anabolic effects of EP4A were seen in mice that received EP4A exclusively in the initial phase. This result might also indicate that EP4A and BMP work cooperatively to stimulate osteoblastic differentiation in its early stage at the interface to the BMP-retaining pellets. Previous in vitro studies support our consideration. Suda et al. reported that EP2/EP4 seems to be involved in osteoblastic differentiation, and EP1/EP3 is likely to be associated with their proliferation [35]. Weinreb et al. described that PGE_2 stimulates osteoblastic differentiation through an anabolic effect in rat bone marrow cultures mediated by activation of EP4, probably
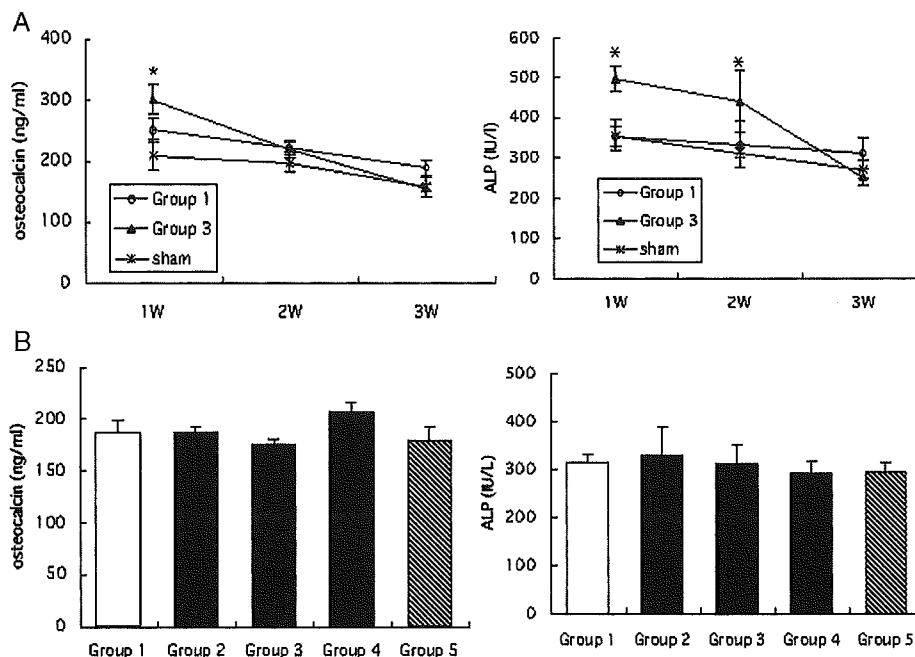
Fig. 7. Serum osteocalcin and ALP. Serum osteocalcin and ALP levels. (A) Serum osteocalcin and total ALP activity from group 3 with rhBMP-2 and ONO-4819 pellets were significantly increased compared to the sham group at 1 week. Total ALP activity from group 3 with rhBMP-2 and ONO-4819 pellets was significantly increased compared to the sham group at 2 weeks. (B) There were no significant differences in serum osteocalcin and ALP levels among the groups at 3 weeks after implantation.

by recruiting noncommitted osteogenic precursors [36,37]. Yoshida et al. described that PGE_2 induced the expression of core-binding factor alpha-1 (Runx2/Cbfa1) and enhanced the formation of mineralized nodules in a culture of bone marrow cells from wild-type mice, both of which were absent in a culture of cells from EP4 knockout mice. EP4 activation increased the number of Runx2 positive cells [30]. EP4 exerts this effect by inducing osteoblast differentiation. On the other hand, several studies indicate that EP4 is essential for PGE_2-induced bone resorption. Suzawa et al. described that, in mouse calvaria cultures, EP4A markedly stimulated bone resorption, and in calvaria culture from EP4 knockout mouse, a marked reduction in bone resorption to PGE_2 was found. EP4A induced cAMP production and the expression of osteoclast differentiation factor mRNA in osteoblastic cells [27]. Stimulation of osteoclastogenesis in cocultures of osteoblasts and spleen cells in response to PGE_2 is markedly decreased when the osteoblasts are derived from cells lacking the EP4 receptor [26–29]. These in vitro studies indicate that PGE_2-EP4 signaling works first in osteoblast precursors to induce osteoblast for bone formation and then works in mature osteoblasts to induce osteoclasts on newly formed bone. Further studies are required to elucidate the detailed mechanism of action of the EP4 receptor agonist in in vitro systems using less differentiated osteogenic cells.

The anabolic effect of PGE_2 on bone was exhibited through the activation of EP2 or EP4 and consequent elevation of intracellular cAMP level [23]. In this respect, the action of an EP4 agonist may be similar to that of PTH,

PDE-4, which also promotes bone formation and intercellular cAMP accumulation. Daily subcutaneous injection of parathyroid hormones (PTH) is known to enhance systemic bone formation, and daily systemic injection of phosphodiesterase-4 (PDE-4)-selective inhibitor, rolipram, can enhance BMP-2-dependent ectopic new bone formation in mice [11,38]. Although the detailed mechanisms of cAMP signal on bone formation have been unclear, these results might indicate that cAMP functionally has a key role in the regulation of the BMP action in osteoblast differentiation, and further studies are required.

Another possible mechanism of the anabolic effect of EP4A on the BMP-induced bone formation comes from studies involving cyclooxygenase-2 (COX-2). Zhang et al. showed the complementary effect of BMP-2 in a bone marrow cell culture from COX-2 knockout mice and suggested that BMP-2 is a target gene for PGE_2-induced bone formation [39]. Chikazu et al. reported that BMP-2 transcriptionally induces COX-2 expression, which in turn regulates, via the Runx2 binding site, production of PGE_2 and promotion of osteoblastic differentiation [40]. These results indicate that BMP and PGE_2 might have complementary or cooperative anabolic effects on mesenchymal cells to stimulate the early phase of osteoblastic differentiation.

Potent bone anabolic activity of EP4A is expected from clinical application for fractures and bone defects in patients. Development of a more effective way of exposing responding cells and tissues to EP4A is likely to be needed for cost effectiveness, clinical efficacy, and long-term safety.

In cases with a longer fracture healing time, such as in humans, a carrier might be necessary for the sustained release of EP4A to be effective. The property of this polymer would allow retention of rhBMP-2 for a period that is significant to elicit new bone formation and thereby provide a scaffold for further bone growth. Retention of the proteins at the implantation site for a sufficient period to promote progenitor cell migration and cell proliferation has been shown to enhance osteoinductive activity. Our results show that local administration of ONO-4819 using PLA–DX–PEG polymer can mimic the local bone anabolic effect of PGE_2 without an excessive dose. The ability to deliver a molecule so that it will induce a specific biologic effect is critical to the success of pharmacological agent therapy.

In conclusion, a new EP4 receptor agonist compound (ONO-4819) can enhance the bone-inducing activity of rhBMP-2 when administered using a local polymer-based carrier with no apparent systemic adverse effects. This compound may be a useful tool for enhancing the perform-ance of rhBMP-2. This could have a significant impact on the costs associated with using this therapeutic cytokine for bone regeneration and repair in clinical practice. Further safety checks are required before ONO-4819 can be used for this purpose.

Acknowledgments

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