【手術のコツ，注意点】

①術中にケージを挿入する際，左側方向からのケージ挿入となる。とくに2つのケージを挿入する場合には，できるだけ正確な側方向からのケージ挿入が望ましい。そのために手術体位をとる際には，イメージにて椎体のendplate像がずれていないか，椎体後縁が二重に写っていないかを十分に確認し，真側面像をモニターできるように工夫しておく。

②術中は皮切部からのCO_2ガスの漏出があると，視野が確保しにくくなる。その際には再度CO_2ガスが後腹膜腔に充満するのを待って，十分に術視野を確保してから作業を進める。

③持続的に腸腰筋を圧排すると，genitofemoral nerveの支配領域に術後疼痛が生じることがあるため，圧排する必要のないときは，これを弛めるようにする。

平均手術時間，出血量など

内視鏡手術は二次元画像を見ながら操作を進めるため，learning curveが存在する。施行当初は内視鏡下手技に時間を要したが，徐々にその時間は短縮し，現在では約3時間程度で後方からの同時手術が可能となった。出血量は平均179.0 m*l*で，後方からの手技と比較して有意に少なかった。

術後の注意点，後療法

術直後は腸管の動きに注意をはらい，腸管蠕動音が微弱であるときには蠕動を亢進するよう薬剤を投与する。現在本術式の後療法はクリニカルパスを作成し，これに準じて行っており，術後3日目に離床し，2週目に退院している。

術後成績

著者らはこれまで28例の変性すべり症に対して，後腹膜鏡視下前方固定術を施行してきた。

当初の10例は後方からの除圧術とペディクルスクリューを用いたインストゥルメンテーションを先行させ，約3週間後に前方から後腹膜鏡視下前方固定術を施行した(第1群)。次の10例に対しては，後腹膜鏡視下に椎間ケージを斜め方向に1つ挿入した後，一期的に後方から片側進入両側除圧術，両側椎間関節のPLLAスクリューによる固定術を施行した(第2群)。また，21例目以降の症例に対しては，後腹膜鏡視下に前方に2つのケージを挿入し，後方からは片側進入両側除圧術のみを施行した(第3群)。

経過観察期間は平均で第1群62.1カ月，第2群31.9カ月，第3群11カ月であった。JOAスコアの改善率はそれぞれ78.4±8.8％，74.7±16.0％，76.7±11.6％で各群間の差は認められなかった。X線学的検討では，すべり率は第1群で術前平均17.0％が術直後11.1％に矯正され，経過観察時12.2％に変化した。第2群ではこれらの値は16.9％，10.9％，13.7％と推移し，第3群では15.8％，11.1％，11.2％と推移した。第3群の代表的な1例の術前後X線像を**図10**に示す。

考察

内視鏡視下手術は，整形外科領域でもとくに後方法を主として施行されるようになっている。著者らは，腰椎変性疾患に対して後腹膜鏡視下に前方固定術を施行してきた。本法では大血管への操作が不要で，腰椎の力学的構成要素である前縦靱帯，後縦靱帯を温存でき，また後方要素では，力学的安定性に最も関与している椎間関節を温存できる。手技への習熟には訓練を要するが，後方からの椎体間固定術と比較して，その手術的侵襲は少なく，今後内視鏡や周辺機器の発達とともにさらなる発展が期待できる。

症例提示

第3群の代表的症例を**図10**に示す。

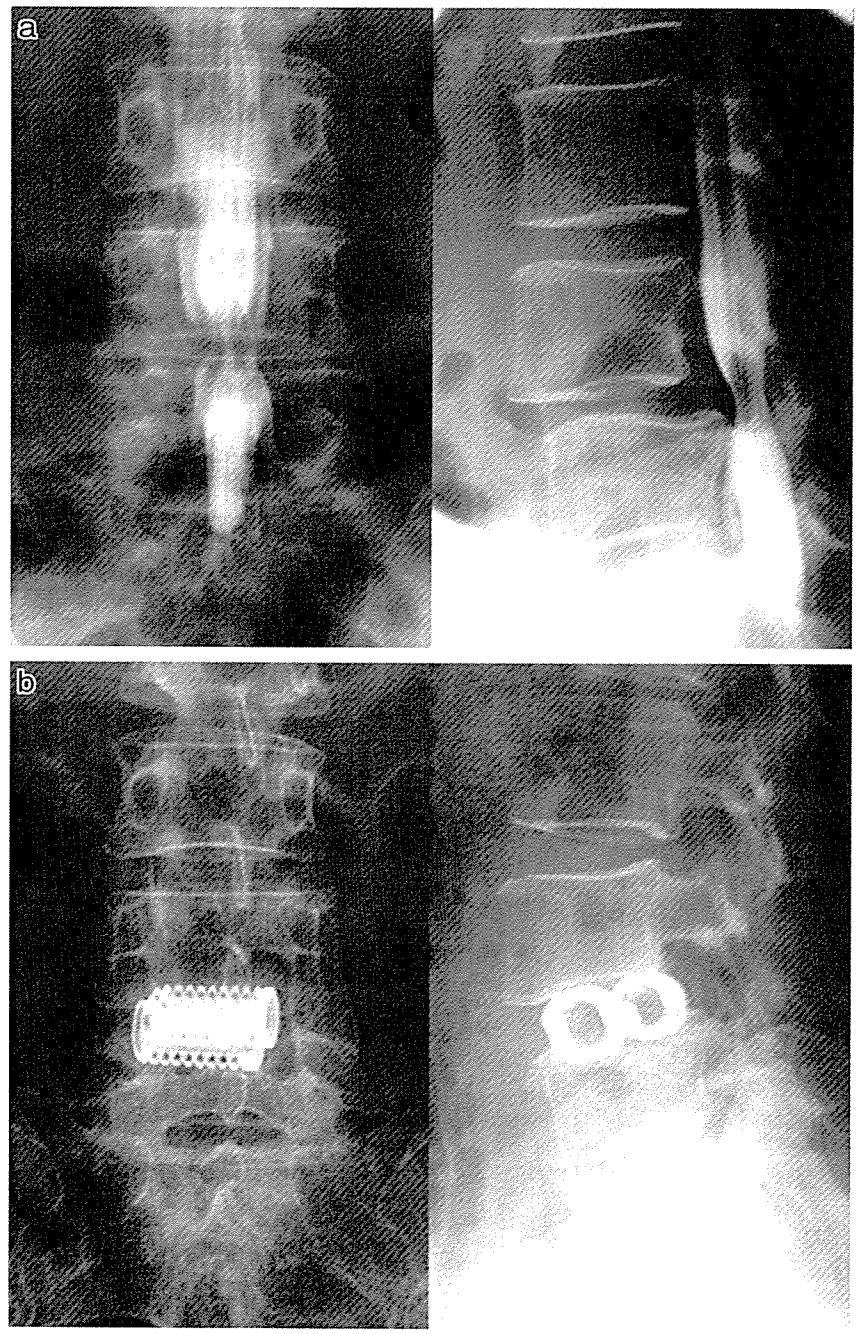

図10　症例
54歳，女性。
a：L4の変性すべりがみられ，同部で硬膜管は圧排を受けている。
b：前方に径16mmのケージ，後方に14mmのケージを挿入し，前弯の形成を図った。

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骨再生医療と骨形成蛋白（BMP）

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骨再生医療と骨形成蛋白（BMP）*

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はじめに

整形外科領域では骨欠損部修復や骨折後偽関節部の骨再生促進法として，自家骨移植術をしばしば行う．自家骨移植が現時点でのもっとも効果的な骨再生促進法であることによる．しかし，自家骨移植に伴う合併症（採骨のための新たな手術創，採骨部の疼痛，知覚鈍麻，変形など）や採骨量に限界があることなどから自家骨移植にかわる，より効果的な骨再生促進技術が求められている．

昨今，整形外科領域の手術において小皮切や内視鏡併用による低侵襲手術（minimum invasive surgery）の発展にみられるように，骨再生についても採骨しない低侵襲かつ効果的な方法が望ましい．そのためにセラミックやハイドロキシアパタイトなどの骨移植代替材料の開発が盛んに行われ，整形外科手術における骨充填材料として受け入れられている．しかし，これらの材料は骨伝導能を有するものの，自家骨移植にみられる骨形成促進作用は期待できないため，骨再生能力に限界がある．もし，これらの人工材料に骨形成促進活性を付与できれば，自家骨に勝る生体材料となるであろう．そのような骨形成促進効果が期待できるのが骨形成蛋白（bone morphogenetic protein：BMP）を用いる方法である．現在BMP（BMP-2，BMP-7）は遺伝子組換えによって合成され供給可能となっている．現時点での問題点はBMPをいかに有効に生体材料と複合するかである．

本稿ではBMPについて解説し，BMPを用いた骨再生医療の近未来的な可能性について論述する．

1 BMPとは

BMPは骨芽細胞系細胞で産生，分泌されている一群の生理活性ペプチドである．その生物学的活性の特徴は，未分化間葉系細胞に作用して軟骨細胞または骨芽細胞へ分化誘導することである．歴史的にはBMPは1965年にUrist[1]によって塩酸脱灰した骨基質に存在することが発見され，その後長期の研究発展の結果，1988年Wozneyら[2]によって2種類のBMP（BMP-2，BMP-4）遺伝子（cDNA）がクローニングされて，その分子構造が明らかになった．その後，生命科学基礎的研究の発展に伴いBMPは*in vivo*での異所骨形成能や*in vitro*での未分化間葉系細胞から骨芽細胞への分化促進作用以外に，個体発生過程での体軸決定をはじめほかのさまざまな臓器の形成過程にも関与していることが明らかにされている[3]．出生後の骨折治癒過程でも，局所的に遺伝子発現の亢進によって仮骨形成に関与していることが報告されている[4,5]．

BMPの骨形成能を臨床的に応用するための研究としては，BMPの遺伝子導入や細胞工学的な方法への応用も考えられる．しかし研究の進捗状況からみて，BMP遺伝子産物である遺

Key words：bone, BMP, drug delivery system

* Bone regeneration by bone morphogenetic protein (BMP)

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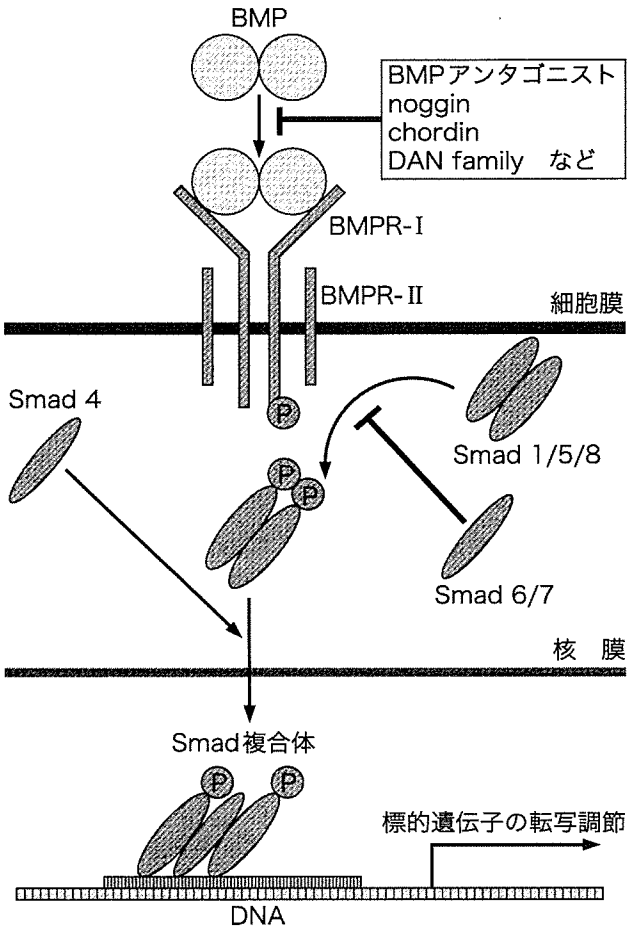


図 1. BMP のシグナル伝達．細胞膜上にある BMP 受容体（BMPR-Ⅰ型および BMPR-Ⅱ型）に BMP が結合すると，Ⅰ型レセプターがリン酸化され活性化される．活性化されたⅠ型レセプターにより R-Smads（Smad 1/5/8）のリン酸化が起り，引き続いて Co-Smads（Smad 4）と複合体を形成して核内に移行，ほかの転写因子や転写共役因子とともに標的遺伝子の発現を誘導する．I-Smads（Smad 6/7）は R-Smads のリン酸化を阻害して BMP のシグナル伝達を抑制する．

伝子組換え体 BMP（recombinant BMP：rhBMP）を用いるのがもっとも簡便で有望である．欧米諸国ではすでに脊椎固定術や偽関節など限定された対象に BMP の使用が認可され，臨床応用が開始されている[6]．しかし，その使用については BMP を局所的に作用させるのに適した薬物伝達系（drug delivery system：DDS）や BMP 使用量の至適化などの問題が残されており，今後は BMP のさらに有効かつ安全な使用方法を確立することが重要な課題である．BMP の効果的使用法が確立されれば，自家骨移植にかわる新しいタイプの骨形成手段が得られ，偽関節の治療，脊椎固定術に限らず腫瘍切除後の骨欠損再建，人工関節再置換での骨欠損部の修復など整形外科領域での広い範囲に応用可能となるであろう．

2 BMP 分子の特性とシグナル伝達

広く臨床利用される可能性が高い BMP は一群の BMP 分子群のうち BMP-2，BMP-7 である．その分子量は 30 K 前後の 2 量体の中性蛋白である．分子内に cystine-knot 構造があり，2 量体分子の構造はきわめて安定である．たとえば 90°C，15 分間の熱処理でも生物活性は保たれる．

BMP は標的細胞の細胞膜上に存在する BMP に特異的なⅠ型とⅡ型の 2 種類のセリン/スレオニン型受容体（BMPR-Ⅰ，BMPR-Ⅱ）を介してそのシグナルを細胞内に伝達する．BMP がⅠ型およびⅡ型受容体と結合し複合体を形成すると，細胞内伝達物質である Smad のリン酸化が起る．現在までに哺乳類では 8 種類の Smad が同定されており，その役割によって特異型 Smad（receptor regulated Smads：R-Smads），共通型 Smad（commom mediator Smads：Co-Smads），抑制型 Smad（inhibitory Smads：I-Smads）に分類されている．BMP の結合により活性化されたⅠ型受容体により，R-Smads である Smad 1/5/8 がリン酸化され，引き続き共通型 Smad である Smad 4 と複合体を形成し細胞質から核内に移行する．Smad の複合体は核内で種々の転写因子と結合，あるいは直接的に DNA と結合することにより BMP 標的遺伝子の転写が活性化されると考えられている[3]（図 1）．最近，骨芽細胞分化においては MAPK（mitogen-activated protein kinase）など，Smad を介さない経路の存在も指摘されている[7]．

3 BMP の DDS

BMP を用いて骨折部や骨欠損部の修復を促進させたり脊椎固定術に用いたりする場合には，BMP を目的の局所にとどめて有効に作用させる工夫が必要である．これによって BMP の有効利用が可能となり，BMP 使用量も少なくできる．

ラット大腿骨骨折モデルを作製し，80 μg も

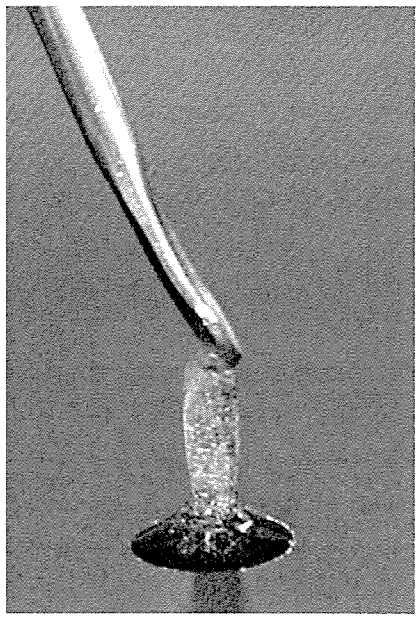
の比較的高用量のrhBMP-2を局所注射することで骨折治癒促進効果が認められたという報告がある[8]．しかしこの用量でも再現性が得られない．一方，ラット大腿骨骨欠損モデルにおいてリン酸カルシウムセメントを担体（キャリアー）として用いた場合，比較的低用量（6.28 μg）のBMPで完全な骨修復が得られたと報告されている[9]．このようにBMPの効果を再現性よく得るためには，BMPを局所にとどめて徐放し，かつ骨形成の足場となる担体との複合使用が必要であることが知られている．すなわち，適切なBMPのDDSが必要であることを意味している．この担体の選択がBMPの臨床応用にさいして大きな問題となってきた．担体物質に求められる特性として，①生体親和性がよく異物反応，炎症反応を起さないこと，②一定時間に生体内で吸収されること，③免疫原性がないこと，④BMPの骨誘導活性を損なわないこと，⑤可塑性があり，BMPによって形成される骨の形状制御をしやすいことなどがあげられる．これまでさまざまな物質がBMPの担体として実験的に研究されてきたが，一般的にはウシ脱灰骨基質やウシ由来精製Ⅰ型コラーゲンが使われてきた．ヒトの脊椎固定に米国ではウシ由来精製Ⅰ型コラーゲンをBMPの担体として用いている．しかしⅠ型コラーゲンはウシ由来の蛋白であり，ヒトに用いると低いながらも抗原性があること，BSE（bovine spongiform encephalopathy）などの病原体混入の危惧があること，力学的強度に欠けることなどの問題点があり，それにかわる理想的な担体の開発が望まれてきた[10]．

4 BMPのための新しい合成DDS

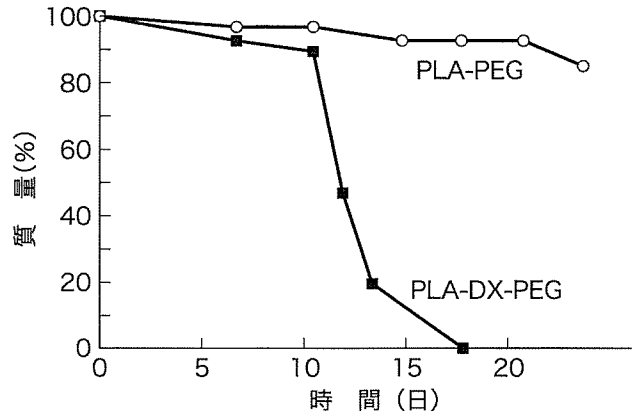
われわれは生体内分解性を有する合成担体（高分子化合物，ポリマー）を開発し，動物実験に用いてその有効性を示してきた．この合成担体はポリ乳酸ポリエチレングリコールブロック共重合体（PLA-PEG）であり，生体内で分解，吸収される．さまざまな組成や分子量の組合せのPLA-PEGポリマーを用いてBMPの担体としての至適化を行った結果，分子量が9,500，組成がPLA：PLGで68：32の化合物がもっとも骨形成に適していることを明らかにした．PLA-PEGの*in vitro*，リン酸緩衝液中の分解特性は時間経過と比例する[11]．さらに分解速度を速めるために，ポリ乳酸鎖にジオキサノンをランダムに組み込んだ共重合体ポリ乳酸-パラジオキサノン-ポリエチレングリコール共重合体（PLA-DX-PEG）を開発した．PLA-DX-PEGはリン酸緩衝液中では17日で完全に分解される．これを用いることによってBMPの徐放速度を適正化できるようになり，さらに有効な骨形成が得られるようになった（図2）[12]．このようなBMPのDDSを単独，またはほかの生体材料と複合して用いることによって，より低用量のBMPで効率的で再現性のよい骨形成が可能となっている．家兎やイヌの長管骨欠損モデルや脊椎固定（後側方固定）モデルでのBMP・ポリマー複合体の有効性についてはすでに報告している[13~15]．

5 BMPでの骨再生医療に残された問題点と未来

巨大骨欠損や偽関節など骨再生活性を強く賦活することが必要なときにBMPを有効に利用すれば，骨移植なしに骨再生修復が可能であることはほぼ間違いない．しかし現時点でBMPが汎用されるにいたっていないのは事実である．BMPの利用が遅れている理由がいくつか存在している．第一にヒトはBMPに対する応答性が低いために多量のBMP（rhBMP）が必要であり，その結果高価な治療法となっている点である．その解決策として，優れたDDSを開発してBMP用量を下げる工夫，さらにBMPの骨誘導活性を増幅する薬剤を開発することによるBMPの低用量化，BMP合成法改良によるBMP自体の低コスト化などがある．これらの問題が解決されれば，BMPによる骨再生技術は広く普及するものと期待される．われわれはすでに上記ポリマーと生体吸収性であるβ-リン酸三カルシウム（β-TCP）の混合体を担体とすることでBMP低用量化が可能であることを報告している（図3）[16,17]．さらにBMPの生物活性を増強する薬剤としてphosphodiesterase阻害薬[ペントキシフィリン(Rolipram)][18]やプロスタグランジンE2(PGE2)の受容体の1つであるEP4のアゴニスト



a．PLA-DX-PEG．無色透明な高分子化合物．室温では粘稠である．



b．37℃，リン酸緩衝液中での分解性を示すグラフ．PLA-DX-PEG は 17 日間で完全に分解される（文献 12 より引用）．

図 2．生体内分解性を有する合成担体

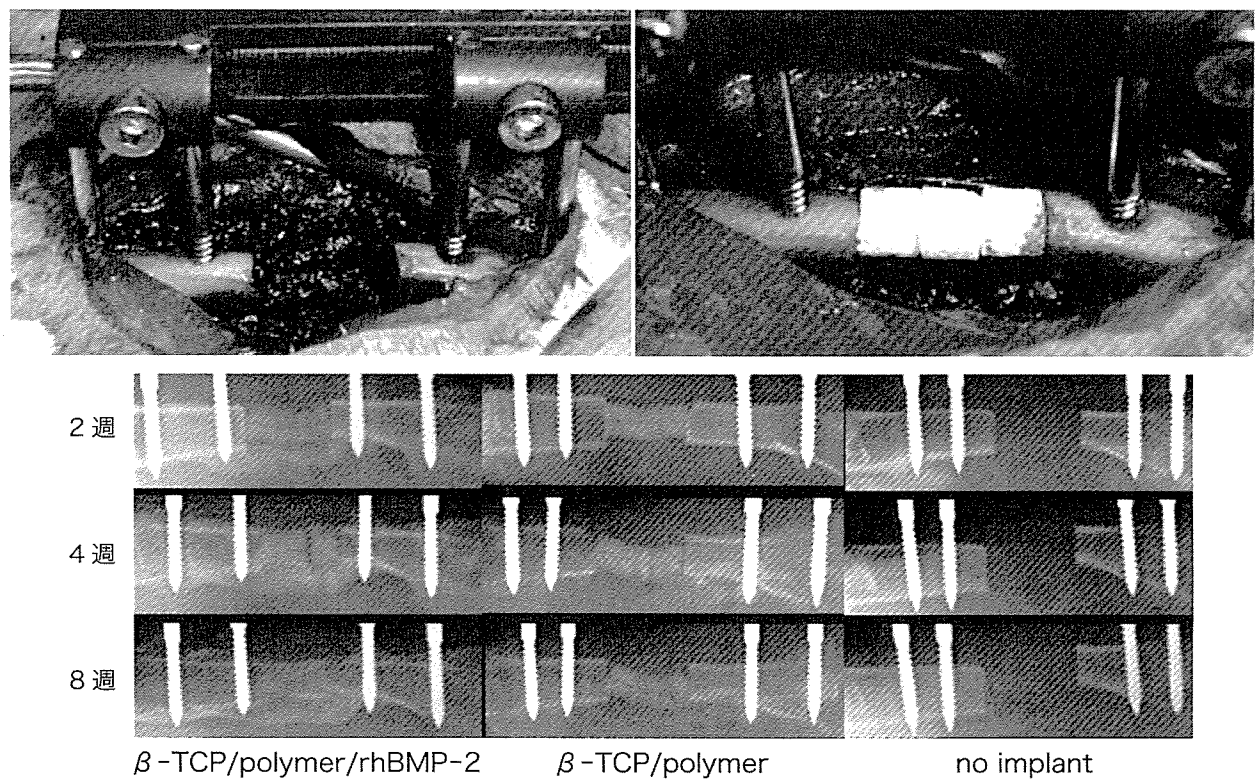
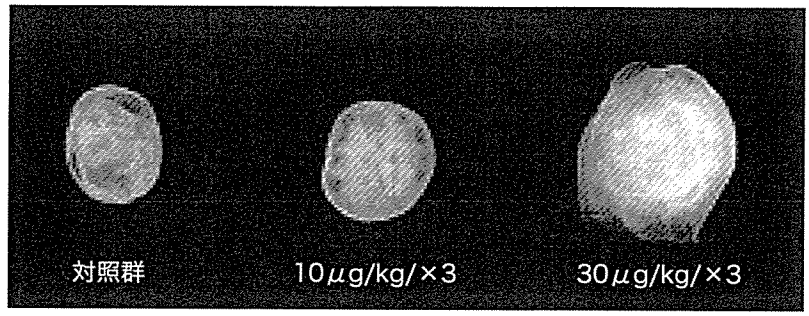
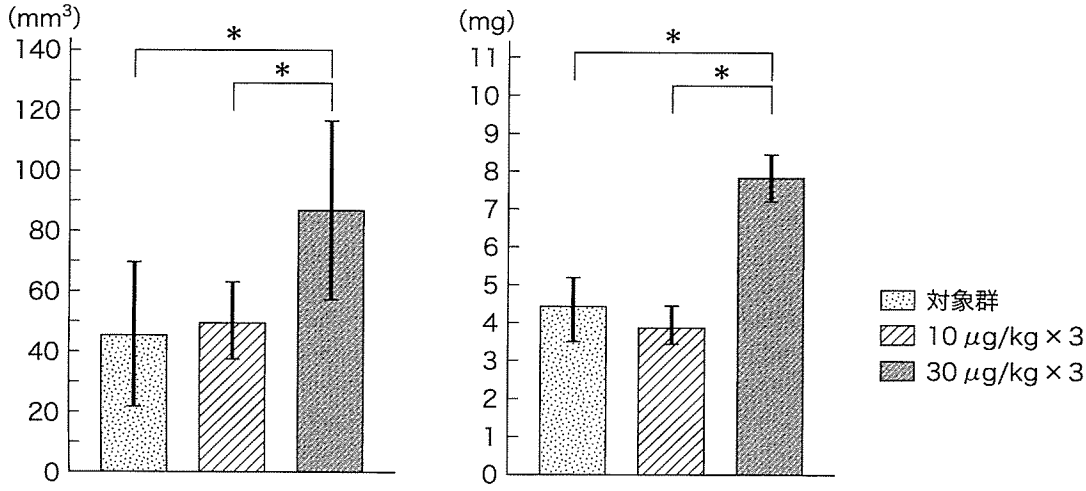


図 3．家兎大腿骨欠損モデルにおける β-TCP/Polymer/rhBMP-2 複合体による骨欠損の修復．大腿骨中央部で長さ 15 mm の骨欠損を作製し創外固定を設置し，骨欠損部に rhBMP-2 を含んだインプラント（β-TCP 直径 5×5 mm 3 個，PLA-DX-PEG 250 mg，rhBMP-2 50 μg）を移植する（上段）．rhBMP-2 を含まないもの，欠損部のままにしておくものを比較対照群とした．BMP 使用群では 4 週までに仮骨形成が認められ，術後 8 週で新生骨による欠損部の完全な架橋が認められる（下段）．修復された長管骨は創外固定をはずしても，長期間にわたり形状・機能ともに保たれている（文献 17 より引用）．



a．軟X線像



b．異所骨の体積　　**c**．骨塩量（DXA）

図 4．BMPによる異所骨形成に対するプロスタグランジンE EP4アゴニスト（ONO-4819）の効果．rhBMP-2含有コラーゲンペレットをマウス背筋筋膜下に埋植し，異所骨の形成を調べる実験系においてプロスタグランジンE EP4アゴニスト（ONO-4819）を皮下注投与したさいの骨形成能への影響を調べた．1日3回30 μg/kg投与により異所骨のサイズ，骨塩量ともに増大している（文献20より引用）．

（EP4A，ONO-4819）［図4］[19,20]，ヘパリン[21]などがBMPの活性を特異的に増幅する作用があることを確認している．その他いくつかのBMP活性増幅作用の有する薬剤の存在が示されている．これらとBMPを併用することによってBMPの低用量化と骨形成作用のさらなる促進が期待できる．

さらに将来，広くBMPの臨床応用を発展させる方向性として，rhBMPの利用以外にBMP遺伝子を用いた遺伝子治療も可能性がある[22]．しかし現在のところは遺伝子導入のための安全かつ有効な発現ベクターを模索しているのが実状であり，まだ骨再生の領域では実用には時間が必要と思われる[23]．

*Ex vivo*で患者の未分化細胞を培養で増殖させ，BMPによって軟骨や骨芽細胞に分化誘導して移植する方法，いわゆる組織工学（tissue engineering）に関しても研究が行われており，理論的には実現可能である[24]．しかし実用化の面からみると大がかりな設備，厳重な無菌環境での培養やそれに要する時間と手間，安全性，経済性，有効性などの面で多くの問題があり，実用化が困難ではないかと危惧される．近未来的にはrhBMPの有効利用による臨床応用が遺伝子治療や組織工学方法に先駆けて汎用されることになるものと推測される．

おわりに

BMPについての基礎的知識としてBMP分子の特性，シグナル伝達制御機構，BMPの実用化のためのDDS，BMP活性増幅方法，さらに遺伝子治療，組織工学への応用の可能性について私見を混ぜて述べた．

今後のBMPの研究発展によって新しい骨再

生技術の確立が可能となることは疑いないものと考える．それによって自家骨移植が不要となり，治療期間短縮とともに能率よい骨再生が可能となって，高度な整形外科治療が実現できることが期待できる．

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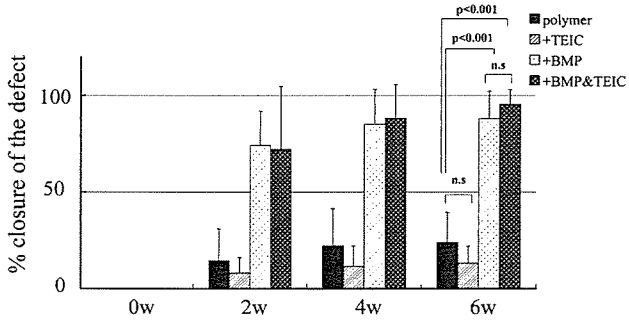

Figure 4. The closure size of critical defect on 3D CT image. The defect area was reduced up to 70% after treatment with rh-BMP2.

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

DISCUSSION

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

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

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

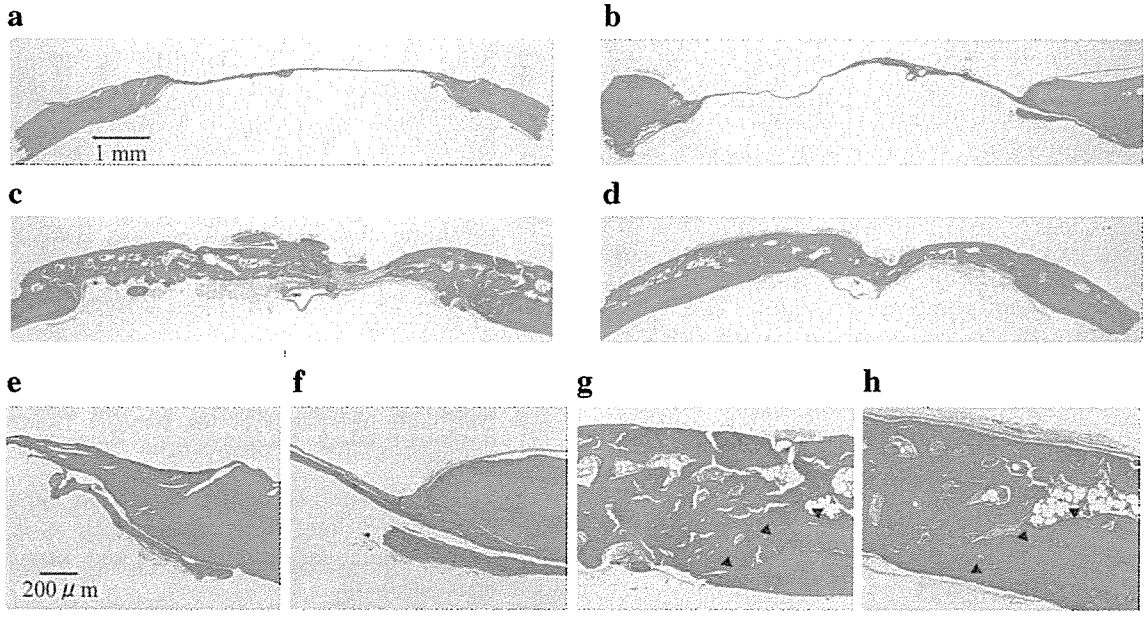

Figure 5. Histology of rat calvarial specimens 6 weeks after treatment with only polymer (a,e), with teicoplanin (b,f), with rh-BMP2 (c,g), with rh-BMP2 and teicoplanin (d,h). Figures e through h are higher (original) magnification (×10) images of the edge between the defect area and normal calvaria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

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

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

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

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

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Abstract

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

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

Introduction

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

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

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

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

Materials and methods

Reagents

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

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

Cell culture

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

Determination of alkaline phosphatase activity

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

Determination of intracellular cAMP

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

Northern blot analysis

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

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

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

Quantitative real-time reverse transcription polymerase chain reaction

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

Western blot analysis

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

Statistical analysis

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

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

Results

ALP induction by BMP-4, PeTx and dbcAMP

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

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

Smad6 mRNA induced by BMP-4 stimulation

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

Suppression of Smad6 expression by cAMP

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

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

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

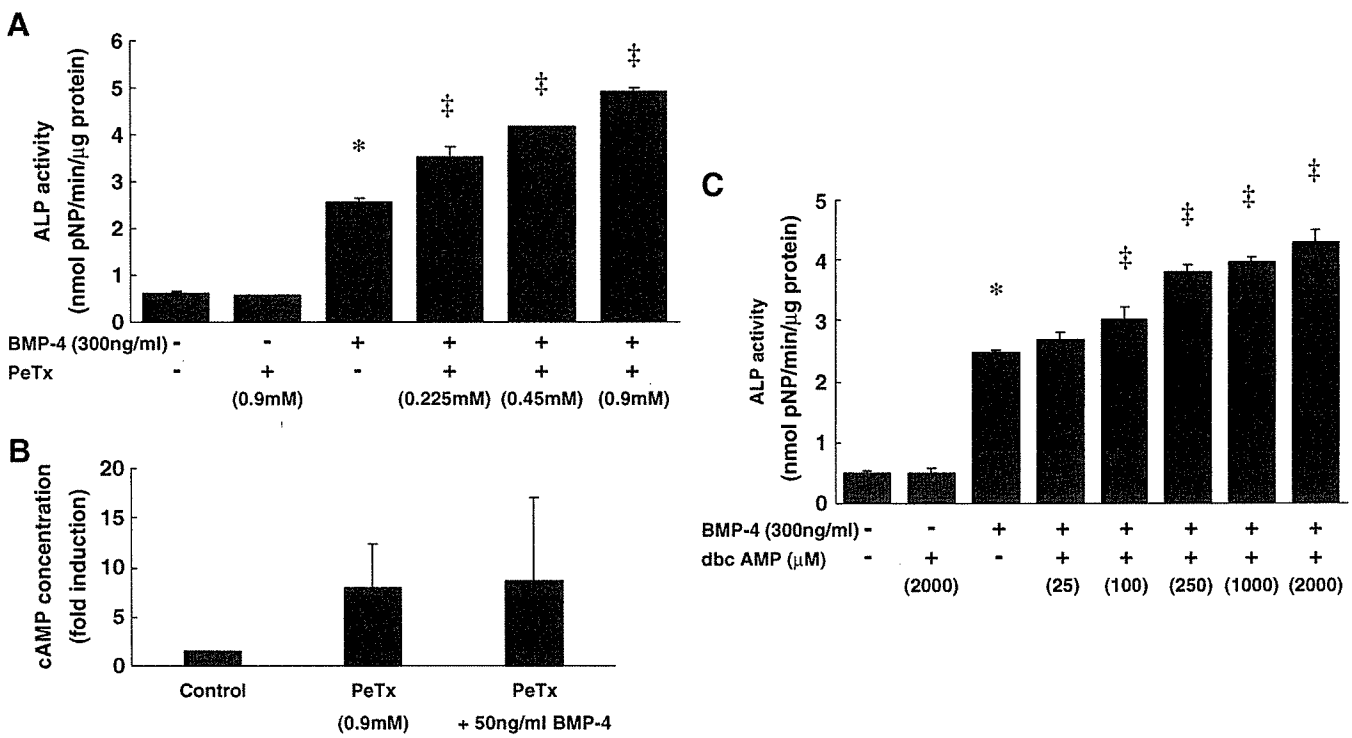

Fig. 1. (A, C) PeTx and dbcAMP intensified BMP-4-induced ALP activation in a dose-dependent manner. ST2 cells were cultured in 24-well plates until confluence then treated with BMP-4, PeTx (A) and dbcAMP (B). After 3 days, ALP activity was measured. (B) Concentration of cAMP in ST2 cells treated with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

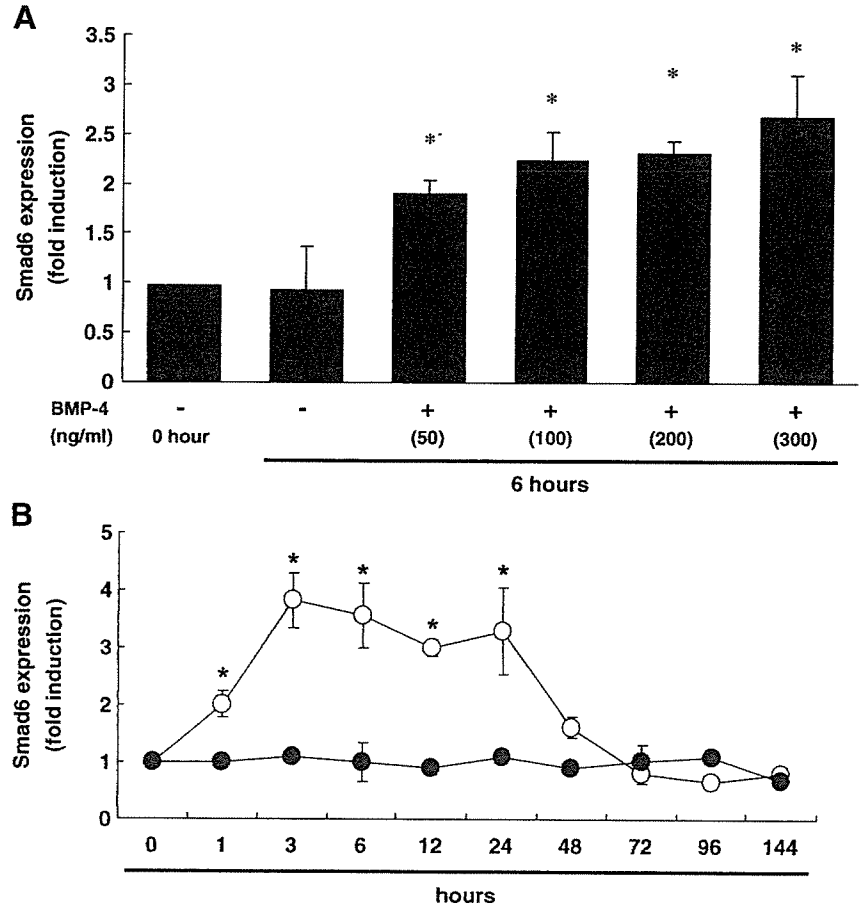

Fig. 2. Effects of BMP-4 on expression of Smad6 in ST2 cells. ST2 cells were cultured in 6-well plates to confluence then treated with BMP-4. After 6 h (A) or other designated times (B), total RNA was extracted, and levels of Smad6 expression were analyzed using real-time RT-PCR, normalized to GAPDH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

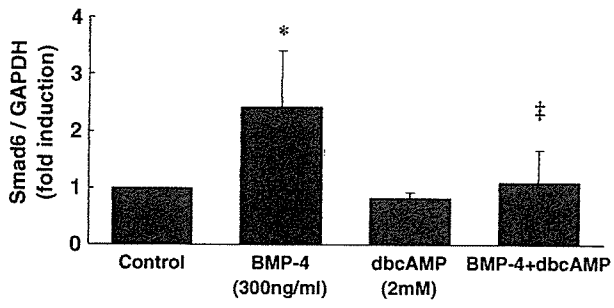

Fig. 3. Cyclic AMP reduced BMP-4-induced Smad6 expression in ST2 cells. Results of Northern blotting. After 24 h of treatment using BMP-4 (300 ng/ml), dbcAMP (2000 μM) or both, mRNA was extracted and analyzed. Messenger RNA obtained using a Poly(A) Quik mRNA Isolation Kit was electrophoresed in agarose-formaldehyde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

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

Prolonged phosphorylation of BMP-specific R-Smads by dbcAMP

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

Discussion

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

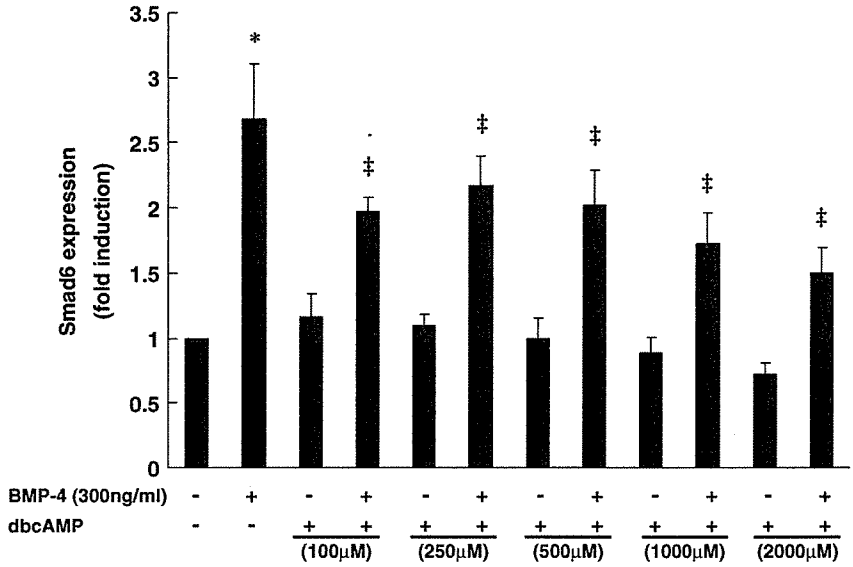

Fig. 4. Cyclic AMP reduced BMP-4-induced Smad6 expression in ST2 cells in a dose-dependent manner. After 12 h of treatment with BMP-4 (300 ng/ml), dbcAMP or both, total RNA was extracted, and expression of Smad6 was analyzed using real-time RT-PCR, normalized to GAPDH expression and presented as the expression level relative to that in control untreated cells. Bars and lines represent mean ± SD for 3 wells. $^{*}P < 0.05$, cells treated with BMP-4 compared with untreated cells. $^{\ddagger}P < 0.05$, cells treated with BMP-4/dbcAMP compared with BMP-4 treated cells.

distinct genes and hydrolyze cAMP and/or cyclic guanosine monophosphate (cGMP), thereby contributing to the regulation of intracellular cAMP levels [14]. A number of compounds inhibiting respective PDE subfamily enzymes in a selective or non-selective manner have already been developed [15]. Interestingly, some PDE inhibitors have been reported to stimulate osteoblastic differentiation and inhibit osteoclastic differentiation in vitro [16,17]. We have

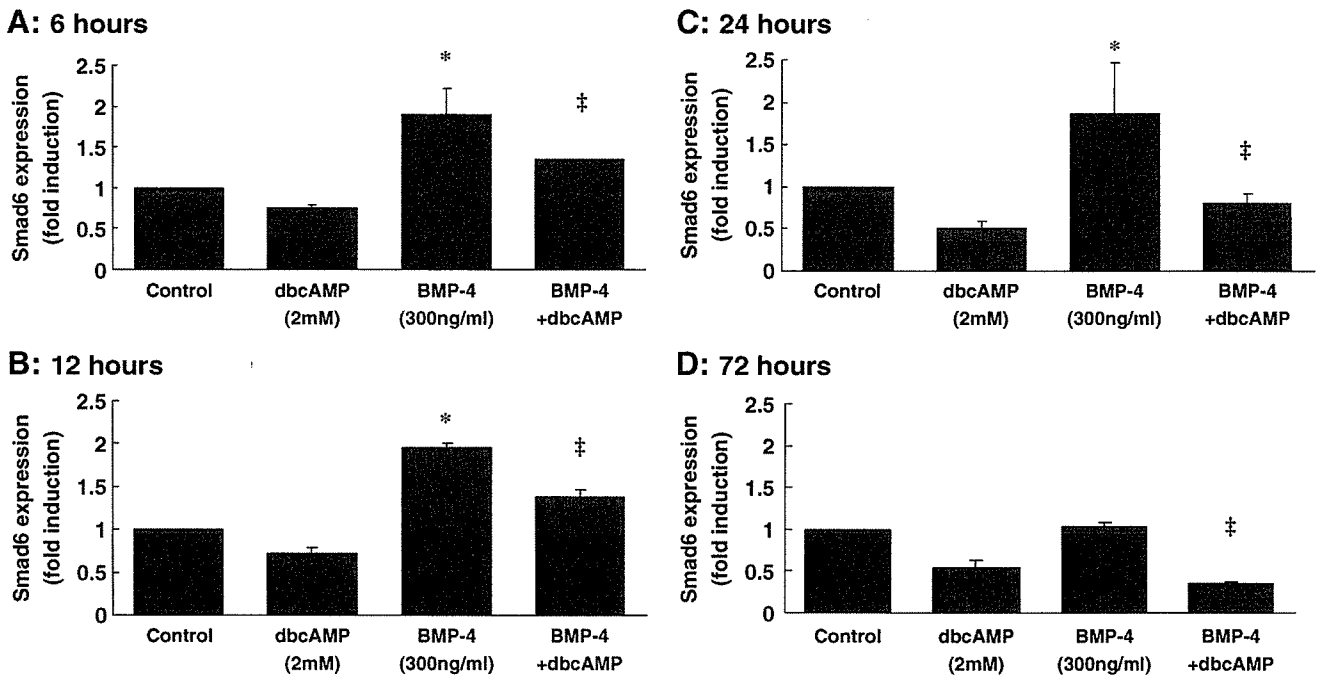

Fig. 5. Cyclic AMP reduced BMP-4-induced Smad6 expression in ST2 cells at 6, 12 and 24 h. ST2 cells were treated with BMP-4 (300 ng/ml), dbcAMP (2 mM) or both for 6 h (A), 12 h (B), 24 h (C) or 72 h (D). Total RNA was then extracted, and expression of Smad6 was analyzed using real-time RT-PCR, normalized to GAPDH expression and presented as expression level relative to that in control untreated cells. Bars and lines represent mean ± SD for 3 wells. $^{*}P < 0.05$, cells treated with BMP-4 compared with untreated cells. $^{\ddagger}P < 0.05$, cells treated with BMP-4/dbcAMP compared with BMP-4 treated cells.

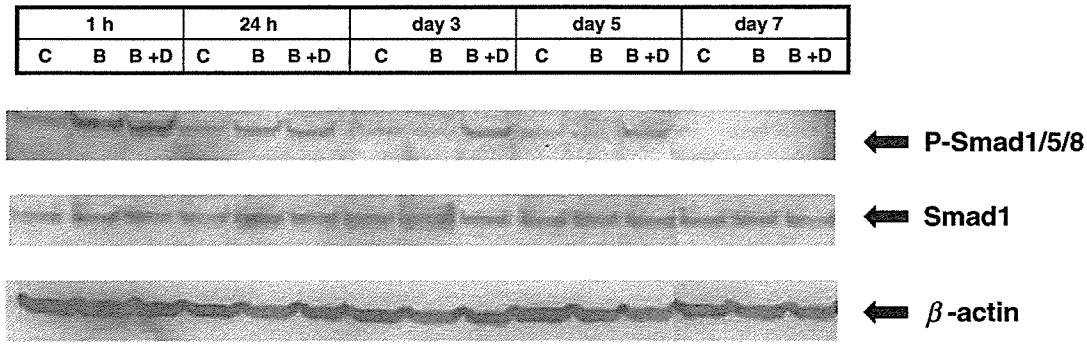

Fig. 6. Effects of dbcAMP on BMP-4-induced Smads signaling in ST2 cells. ST2 cells were pretreated for 1 h in 5% FBS before stimulation. Cells were treated with BMP-4 (300 ng/ml) or BMP-4 and dbcAMP (2 mM). Whole-cell extracts were prepared at indicated time points followed by immunoblotting. Identical amounts of protein samples were applied and run on each lane of a 10% acrylamide gels, blotted to an ECL nitrocellulose membrane, and membranes were developed using ECL plus reagent. Activated BMP-specific R-Smads were detected by anti-phospho-Smad1/5/8 antibody. Bands for Smad1 and β-actin are shown as a loading control. C, control; B, BMP-4; B + D, BMP-4 and dbcAMP.

reported that the PDE inhibitors, PeTx and Rolipram, increase bone mass mainly by promoting bone formation in normal mice [4,18]. Furthermore, PDE inhibitors have been shown to exert therapeutic effects in different experimental osteopenia models [17,19,20]. Although some PDE inhibitors reportedly promote bone formation under both in vivo and in vitro conditions, the precise mechanisms leading to the osteogenic effects of those PDE inhibitors are currently unknown. The present study was performed to gain clues regarding the anabolic effect of the PDE inhibitor PeTx on osteoblastic differentiation of bone marrow stroma cell-derived osteoprogenitor cells, ST2 cells.

Our previous report showed that daily injection of PeTx increased systemic bone mass by enhancing bone formation [18], and it also stimulated BMP-2 induced ectopic bone formation [4]. However, the mechanisms underlying the effects of PDE inhibitors on bone metabolism have remained obscure. Regarding mechanisms of action, one of the basic but major questions has been whether the anabolic effect of PDE inhibitors on bone metabolism is linked specifically to BMP signaling system to induce osteoblastic differentiation or results from functional activation of fully differentiated osteoblasts independent of BMP. Based on the results in our present and previous studies, the effect of PDE inhibitors might be brought about in association with BMP since the effects of PDE inhibitors in enhancing osteoblastic differentiation were not seen in the absence of BMP but were consistently noted in the presence of BMP, indicating a potential function of the PDE inhibitor in enhancing BMP signaling through elevation of intracellular cAMP levels [21]. In addition, intracellular cAMP-elevating agents such as dbcAMP and forskolin also increase BMP-4-stimulated osteoblastic differentiation when estimated by elevating ALP activity [21]. These results indicate that cAMP-elevating agents might enhance BMP signaling pathway to enhance bone formation. Regarding the effects of PDE inhibitors on BMP signaling, the results of a recent study suggest that the anabolic actions of PeTx might be attributable to cross-talk between BMP signaling and protein kinase C (PKC) signaling cascades [22]. Rawadi et al. noted that PeTx could promote osteoblast differentiation not by protein kinase A (PKA) activation, since inhibition of PKA by H-89 (a protein kinase inhibitor) did not alter the PDE-induced osteogenic reaction, but through activation of extracellular signal-regulated kinase (ERK) 1/2 and p38 kinase pathways [22]. However, we could not exclude the possible contribution of cAMP-activated PKA to the acceleration of osteoblastic differentiation by PeTx since inhibition of PKA by H-89 partially abolishes PDE inhibitor-mediated increases in the induction of ALP by BMP-4 (data not shown). Cross-talk between the cAMP/PKA cascade and the Smads-mediated BMP signaling pathway awaits elucidation in future studies.

Among the 3 groups of Smad proteins involved in the BMP signaling pathway, Smad6 and Smad7 are classified as I-Smads. I-Smads interact with activated serine/threonine kinase BMP or TGF-β receptors and compete with R-Smads (Smad1/5/8) to activate the receptors [23]. Smad6 has also been reported to compete with common-partner Smad (Co-Smad, Smad4) in forming complexes with R-Smads. Smad6 might be more crucial in the negative feedback loop as Takase et al. [24] reported that Smad6 mRNA was markedly induced by BMP-2 or BMP-7/OP-1 in various osteoprogenitor cell lines, whereas Smad7 expression remained unchanged in most cells. In this context, Smad6 was targeted to examine involvement of the protein in the mechanism enhancing BMP signaling by cAMP. We confirmed up-regulation of Smad6 expression in response to BMP-4 stimulation in ST2 cells. Interestingly, up-regulated expression of Smad6 by BMP-4 stimulation appears to be partially suppressed by elevated intracellular cAMP levels on addition of cell-membrane-permeable dbcAMP in a dose-dependent manner in ST2 cells. Elevated levels of phosphorylated R-Smads following BMP-4 treatment and dbcAMP lasted longer than that with BMP-4 treatment alone on Western blot analyses. Although dbcAMP significantly suppressed the

expression of Smad6 mRNA induced by BMP-4 within 6 h (Fig. 5A), levels of phosphorylated R-Smads induced by BMP-4 at 24 h were not altered (Fig. 6). This discrepancy might be explained by following two points. First, Smad6 was evaluated by mRNA level, whereas phosphorylated R-Smads were determined by protein level. Second, Smad6 binds in a stable manner to serine/threonine kinase receptors and then interferes with phosphorylation of R-Smads by receptors. This negative feedback loop might require the appropriate time lag. These results suggest that the anabolic effects of cAMP on BMP-4-induced osteoblastic differentiation might be partially attributable to suppressed expression of Smad6 in the negative feedback loop and the facilitation of positive BMP-4 signaling in cells. In addition, dbcAMP enhanced the expression of Id-1 mRNA, an inhibitor of myogenesis, induced by BMP-4 (data not shown). These findings taken together suggest that cAMP might be a modulator of BMP signaling. Ishida et al. identified the 28-base pair regions responsible for transcriptional activation by BMPs in the mouse Smad6 promoter [25]. Ionescu et al. characterized a putative cAMP response element (CRE) site in the adjacent 17-base pair [26]. In that report, dominant negative cAMP response element binding protein reduced BMP-2-stimulated Smad6 gene transcription [26], but these results were not consistent with our current results. Potential causes for these inconsistencies include interactions of other transcriptional factors and differences in cells and BMPs. Similar mechanisms were reported to explain signal cross-talk between BMP and TGF-β [27]. Further studies are needed to elucidate relationships between cAMP and BMPs.

Smad proteins play important roles in BMP signaling and display characteristic pathways. Only a limited number of previous reports have examined relationships between the Smad pathway and cAMP. On addition of dbcAMP, BMP-4-induced Smad6 expression was significantly suppressed (Fig. 4). These results indicate that dbcAMP enhances BMP-4 osteogenic activity through the suppression of a self-regulated negative feedback loop. Parathyroid hormone (PTH) or its amino-terminal fragment is known to enhance systemic bone formation following daily subcutaneous injection and is currently utilized to recover bone mass in osteoporotic patients without the precise mechanisms of action being understood. As PTH also elevates intracellular levels of cAMP in cells with PTH-specific receptors, cAMP interference in the BMP negative feedback might be involved in PTH-enhanced systemic bone formation. Additional studies confirming suppression of I-Smads by PTH treatment are necessary to elucidate the mechanisms of action underlying the anabolic effects of PTH.

In conclusion, suppression of BMP-4-induced Smad6 expression appears to represent one of the mechanisms by which BMP action is enhanced by PeTx and dbcAMP treatments. Manipulation of the BMP signaling loop may also provide new insights into enhancing the efficacy of BMP-mediated local new bone formation for the treatment of damaged bone.

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