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話　題

Fibrodysplasia ossificans progressiva (FOP) とBMP情報伝達異常のup to date*

片桐岳信**

Key Words：fibrodysplasia ossificans progressiva (FOP), bone morphogenetic protein (BMP), osteogenesis, intractable disease, receptor

はじめに

Fibrodysplasia ossificans progressiva (FOP；進行性骨化性線維異形成症) は，以前にはmyositis ossificans progressiva (MOP；進行性化骨筋炎) とも呼ばれていた疾患で，全身の骨格筋を中心に靱帯や腱における異所性骨形成の進行を特徴とする遺伝性疾患である[1] (図1)．FOPでは，患者の成長に伴って多くの関節が癒合して可動域が極度に小さくなるため，病気の進行に伴い自立的な運動がきわめて困難となる．異所性骨化は顔面の関節にも及び，開口障害や嚥下障害を伴う症例も認められる[2]．その一方では，心臓や横隔膜，舌などの筋組織に異所性骨形成を認めた報告はない．

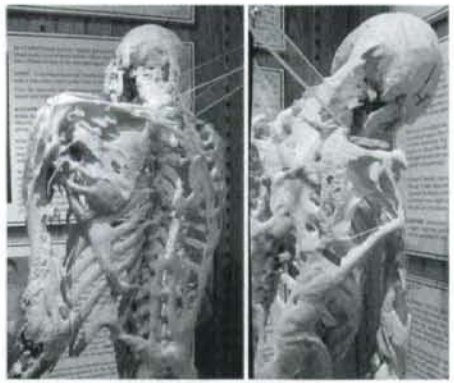

図1　FOPにおける異所性骨形成
米国ペンシルバニア州フィラデルフィアのMutter Museumに展示されているFOPの骨格標本を，許可を得て撮影した．

* Recent progresses in fibrodysplasia ossificans progressiva and BMP signaling.

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現時点で，FOPの異所性骨化を抑制できるような有効な治療法は確立されておらず，異所性骨形成の発症や進行を可能な限り抑えることが重要である．FOPの異所性骨形成は，打撲や転倒などの事故で筋組織に外傷を受けると急激に進行する[12]．また，FOP患者の筋肉内注射やバイオプシー，異所性骨の外科的除去手術など，侵襲的医療行為で骨化が誘発された症例が世界的に多く報告されている[2]．とくにこれまでFOPの確定診断には4年以上を要しており，この間の検査や処置によって骨化が誘発された経験をもつ患者数が多いことは注目に値する[2]．このほかに，親知らずの抜歯後に開口障害を生じた例があり，麻酔を含めた歯科治療に際しても注意が必要とされる．インフルエンザウイルスの感染により骨化が進行した症例も報告されている[2]．

FOPは，従来症例数が少ないこともあり医療関係者に十分に知られていない場合があった．FOPが難病に認定されたことに伴いFOPの理解を深めることは，治療法が確立されていない本疾患において，異所性骨形成の誘発を少しでも遅延させることができる可能性がある．本稿では，最近の研究成果を交えながらFOPを概説する．

遺伝性疾患としてのFOP

FOPの発症頻度は，イギリス・ウェールズ地方における研究結果に基づき約200万人に1人と推定され，明確な地域差は認められていない[3]．わが国の人口を約1億2000万人とすると，この発症率から約60名前後のFOP患者が存在するものと推定される．最近の厚生労働省難治性疾患克服研究事業によって実施された医療機関とFOP患者会を対象としたアンケート調査でも，ほぼ同程度の患者数が報告されている[3]．

現在，国内で生存が確認されているFOPの30数例はすべて孤発性で，FOP患者の両親や兄弟，姉妹にFOPの症状は認められない．一卵性双生児の姉妹にFOPに特徴的な母趾の変形が認められたとする1例を除き，1家系に複数のFOP発症例はない．一方，海外では一卵性双生児の双方に発症した例や，父親から女児を含む複数の子供に遺伝した症例が確認されたことにより，FOPは常染色体優性遺伝の形式を示す遺伝性疾患であることが明らかとなった[4,5]．しかし，症例数が少ないことから，責任遺伝子を同定するために必要な大家系の遺伝子解析は困難であった．

FOPの特徴

FOPの筋組織における異所性骨形成は，出生時にはほとんど認められず，成長に伴って進行する．FOPの出生時の最大の特徴は，左右対称に外反母趾様の変形を有することで，ほぼすべてのFOP症例で確認されるという[1,2]．現時点では，この出生時の外反母趾様の症状と遺伝子診断（後述）で，大部分のFOPを確定診断できると考えられる．

2～5歳前後になると，頭，頸，背中に痛みを伴うflare-upと呼ばれる腫瘤が出現し，数週間にわたって持続する[1,2]．後述するように，FOPではバイオプシーが禁忌とされるために組織学的情報が乏しいが，比較的初期の腫瘤は液状物質や線維芽細胞で満たされており，骨形成を示すような像は認められない．その後，腫瘤の消失に伴いその部位に異所性骨形成が進行する[1,2]．このとき，骨形成は体幹部から末梢，頭部から尾部，近位から遠位と進行する傾向があり，30歳頃までに全身に及ぶ．FOPの異所性骨形成は成長期に著しく進行し，20～30%の患者に難聴が認められる．

筋組織における異所性骨形成

以前から，FOPの発症にはbone morphogenetic protein（BMP）のシグナル異常が関与する可能性が指摘されていた[6]．これは，BMPが他のサイトカインやホルモンとは異なり，筋組織で異所性骨形成を誘導する特別な活性物質であることによる[6]．BMPは骨組織から単離・同定された一群のサイトカインで，これまでに15種類以上の相同性の高いBMPがクローニングされており，それらの一次構造からすべてtransforming growth factor-β（TGF-β）ファミリーに分類される[6]．

筋組織内での異所性骨誘導活性は，TGF-βファミリーのBMP以外の因子には認められず，きわめてBMPに特異的な活性と考えられている．今日では，BMPは局所的な骨形成を促す医薬品としても臨床応用されつつある．

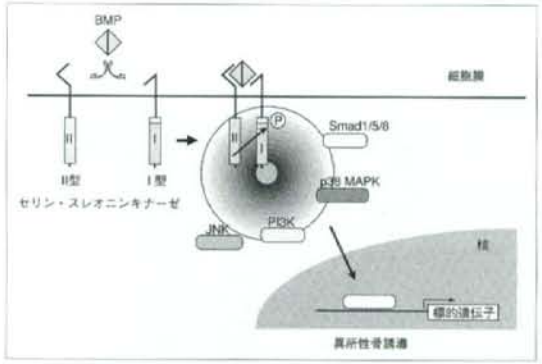

図2　BMP受容体による細胞内情報伝達
BMPは，標的細胞表面のI型とII型受容体に結合する．II型受容体がI型受容体を活性化し，I型受容体がT式の転写因子をリン酸化反応によって活性化する．（筆者作成）

BMPは*in vitro*でも筋芽細胞や線維芽細胞を骨芽細胞や軟骨細胞へ分化誘導する．マウスの筋芽細胞株として広く用いられているC2C12細胞は，筋組織の再生を司る衛星細胞に由来すると考えられており，BMP存在下で培養すると成熟筋細胞への分化が抑制され，代わりに骨芽細胞への分化が誘導される[6]．興味深いことに，筋分化を抑制する因子は数多く知られているが，BMP以外の因子が筋芽細胞を骨芽細胞へと分化誘導した例はない[6]．このことから，BMPに特異的な細胞内情報伝達系が，筋組織内での異所性骨形成を誘導すると考えられる．

BMP受容体の変異とFOP

BMPは，標的細胞表面に存在する構造の異なった2種類の特異的受容体と結合する（図2）．最近の研究から，FOPにおける遺伝的変異は，BMP受容体をコードする遺伝子の1つに認められることが明らかとなった．

2種類のBMP受容体は，どちらも膜貫通型セリン・スレオニンキナーゼで，I型受容体として4種類，II型受容体として3種類の分子が同定されている[6]（図2）．BMPとBMP受容体が複合体を形成すると，II型受容体がI型受容体のGSドメインと呼ばれる細胞内領域をリン酸化し，I型受容体のキナーゼを活性化する．活性化されたI型受容体は細胞質でさらに下流の因子をリン酸化し，最終的に転写因子を介して核内へとシグナルが伝達される．

2006年，Shoreら[11]は，家族性FOPの5家系を用いた連鎖解析から，2番染色体q23-24でBMPのI型受容体の1つALK2受容体をコードする*ACVR1*遺伝子内に，FOP患者に共通の617G>A変異を見出した．この変異は，最終的に7例の家族性FOPと32例の孤発性FOPにも認められた[12]．国内の解析でも，独立した研究によりそれぞれ17例と3例の孤発性FOPで同一の変異が確認されている[12,13]．これらの発見より，FOPの責任遺伝子は*ALK2*と考えられ，この情報を基にしたFOPの遺伝子診断が可能となった．従来，確定診断に4年以上を要したことに比べると，数日の遺伝子診断で正確にFOPを判定できる意義はきわめて大きい．

FOPで同定された*ALK2*遺伝子の617G>A変異は，206番のアルギニン残基をヒスチジン残基に変異させる（R206H）[11]．この部位は，ALK2受容

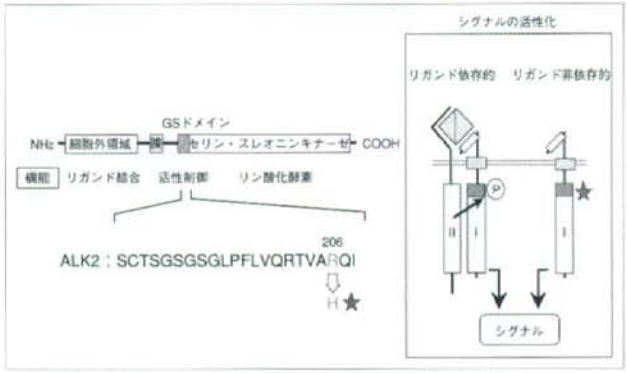

図3　ALK2受容体のアミノ酸置換による活性化とFOPにおける変異
BMPやTGF-βの受容体は，GSドメインの保存された1アミノ酸を置換するとリガンド非依存的に活性化されることが知られていた．FOPで同定されたALK2(R206H)変異は，この隣のアミノ酸であった．最近，ALK2(G356D)変異も同定されている．（筆者作成）

体がII型受容体によってリン酸化されるGSドメイン内に相当する(図3)．すでに10年以上前に，TGF-βファミリー受容体でGSドメインに変異を導入して1アミノ酸を酸性アミノ酸に置換すると，キナーゼが構成的に活性化されることが報告されていた(図3)．ALK2受容体の場合，207番目のアスパラギン残基を酸性アミノ酸に置換すると活性化することが示されていた．FOPで同定された変異が206番目のアルギニン残基であったことから，FOPにおけるALK2受容体の活性化が予想された．

FOPにおけるALK2の活性化

そこで，ALK2(R206H)変異受容体を培養細胞に過剰発現させると，確かに下流のBMP細胞内情報伝達系であるSmad1/5/8のリン酸化と核内への集積が誘導され，BMPシグナルの標的遺伝子のプロモーター活性も上昇した[14][15]．さらに，ALK2(R206H)受容体をSmad1とともに筋芽細胞で発現させると，骨芽細胞への分化が誘導されることが判明した[15]．これらの結果より，FOPで同定されたALK2(R206H)は構成的に活性化されたBMP受容体で，常にBMPの細胞内情報伝達系が活性化されることで異所性骨形成が進行すると考えられた．

ごく最近，国内の1症例で，これまでと異なるALK2遺伝子の1067G>A変異が報告された[16]．この変異はキナーゼ領域内のG356D変異をひき起こす．米国の研究グループも，2006年の米国骨代謝学会において，ALK2のG356Dに加えキナーゼ領域の別の変異を同定したことを発表した[17]．これらの新しい変異が，ALK2の活性などにどのように影響しているか今後の解析が待たれる．

筋再生とFOP

ALK2(R206H)変異受容体は構成的活性型であるが，少なくとも筋芽細胞を用いた骨芽細胞への分化誘導活性は弱い．また，ALK2の発現はさまざまな組織で認められるにもかかわらず，FOPでの異所性骨化は筋組織を中心に認められる．これらは，FOPの異所性骨化がALK2の構成的活性化と，さらに別の機序の相互作用に基づく可能性を示唆する．

先に述べたように，FOPの症例では筋組織の

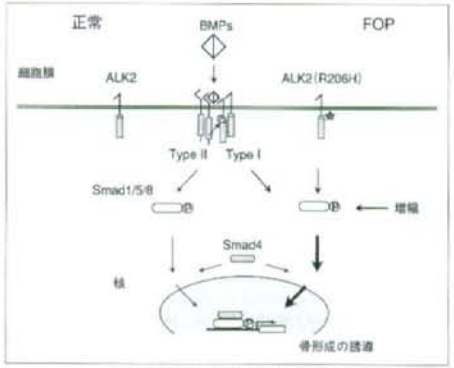

図4　FOPにおけるBMPシグナルの変異
野生型のALK2は，BMPと結合したときのみSmad1/5/8を介して骨形成を誘導する．一方，FOPのALK2(R206H)受容体は，GSドメインの変異によりBMPが結合しなくとも構成的にSmad1/5/8を活性化する．ALK2を阻害する分子は，FOPの治療に応用できる可能性がある．（筆者作成）

損傷が異所性骨化を誘発する[18]．そこで，筋再生に伴ってBMPシグナルを相乗的に増幅する機構が存在する可能性を検討した．その結果，ALK2を含むBMP受容体の発現量は変化しないにもかかわらず，ALK2によってリン酸化される転写因子Smad1とSmad5の発現量が増加することが判明した[16]．ALK2(R206H)を，Smad1やSmad5とともに筋芽細胞で発現させると骨芽細胞への分化誘導活性を示すことから，ALK2の遺伝的な変異に加えて筋再生に伴って転写因子が増加することで，異所性の骨形成が進行する可能性が考えられる(図4)．

FOPの治療

現時点では，主にFOPの腫脹に対してステロイドなどの抗炎症薬，さらに異所性石灰化に対してビスフォスフォネート製剤などが用いられているが，有効性が確立した治療法はない．しかし，少なくとも*in vitro*では，BMP受容体によるSmadシグナルの特異的低分子阻害剤として報告されたdorsomorphinが，ALK2(R206H)受容体による骨芽細胞分化を強力に抑制した．将来，この化合物からFOPの治療薬が開発されることが期待される．

最近，骨髄移植に伴い免疫抑制剤を14年間投与していた興味深いFOPの症例が報告された[19]．この症例では，免疫抑制剤投与中は異所性骨化がまったく起こらなかったものの，骨髄細胞が野生型ALK2の遺伝子型を示すにもかかわらず，投薬中止によって骨化が再発したという[19]．この報告は，骨髄移植ではFOPの骨化を防ぐことはできないことを明確に示すと同時に，ある種の免疫反応が骨化に関与することを示唆する．

おわりに

Fibrodysplasia ossificans progressiva(FOP)は，bone morphogenetic protein(BMP)受容体の機能獲得型変異という初めての例であった．BMPの骨形成における重要性は古くから研究されてきたが，FOPにおける異所性骨化は，改めてBMPシグナルの強力な骨誘導作用を示したものと考えられる．

近い将来，FOPの発症機序が明らかとなり，効果的な治療法が確立されることが望まれる．それと同時に，FOP患者が受診する可能性のある整形外科や小児科，歯科などの医療従事者にFOPの新しい情報を広め，現場との連携を深めることで，現時点で実現可能な最善の診療法を検討することも必要とされている．

文　献

1) Kaplan FS, Shen Q, Lounev V, et al. Skeletal metamorphosis in fibrodysplasia ossificans progressiva. J Bone Miner Metab. In press 2008.
2) Kitterman JA, Kantanie S, Rocke DM, et al. Iatrogenic harm caused by diagnostic errors in fibrodysplasia ossificans progressiva. Pediatrics 2005；116：e654.
3) Scarlett RF, Rocke DM, Kantanie S, et al. Influenza-like virus illnesses and flare-ups of fibrodysplasia ossificans progressiva. Clin Orthop Relat Res 2004；423：275.
4) Connor J, Evans DA. Genetic aspects of fibrodysplasia ossificans progressiva. J Med Genet 1982；19：35.
5) 芳賀信彦，川端秀彦，鬼頭浩史，ほか．日本における進行性骨化性線維異形成症患者の診療状況に関する研究．In：厚生労働省科学研究費補助金難治性疾患克服研究事業，脊柱靭帯骨化症に関する調査研究，平成19年度総括・分担研究報告書．2008. p. 165.
6) Eaton WL, Conkling WS, Daeschner CW. Early myositis ossificans progressiva occurring in homozygotic twins. J Pediatr 1957；50：591.
7) Kaplan FA, McCluskey W, Hahn G, et al. Genetic transmission of fibrodysplasia ossificans progressiva：report of a family. J Bone Joint Surg 1993；75-A：1214.
8) Kaplan FS, Tabas JA, Zasloff MA. Fibrodysplasia ossificans progressiva：a clue from the fly? Calcif Tissue Int 1990；47：117.
9) Katagiri T, Suda T, Miyazono K. The bone morphogenetic proteins. In：Miyazono K, Derynck R, editors. The TGF-β Superfamily Monograph. New York：Cold Spring Harbor Press；2008. p. 121.
10) Katagiri T, Yamaguchi A, Komaki M, et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 1994；127：1755.
11) Shore EM, Xu M, Feldman GJ, et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 2006；38：525.
12) 片桐岳信，福田　亨，神田将和，ほか．Fibrodysplasia Ossificans Progressiva（FOP）におけるBMP受容体ALK2の変異とその機能解析．日整会誌 2006；80：710.
13) Nakajima M, Haga N, Takikawa K, et al. The ACVR1 617G>A mutation is also recurrent in three Japanese patients with fibrodysplasia ossificans progressiva. J Hum Genet 2007；52：473.
14) Fukuda T, Kohda M, Kanomata K, et al. A constitutively activated BMP receptor, ALK2, induces heterotopic bone formation in patients with fibrodysplasia ossificans progressiva（FOP）[abstract]. J Bone Miner Res 2007；Suppl 1：S10.
15) Shen Q, Xu M, Little SC, et al. Activation of BMP signaling by the FOP ACVR1 R206H mutation [abstract]. J Bone Miner Res 2007；Suppl 1：S43.
16) Furuya H, Ikezoe K, Wang L, et al. A unique case of fibrodysplasia ossificans progressiva with an ACVR1 mutation, G356D, other than the common mutation（R206H）. Am J Med Genet A 2008；146A：459.
17) Shore EM, Xu M, Connor JM, et al. Mutations in the BMP type I receptor ACVR1 in patients with fibrodysplasia ossificans progressiva（FOP）[abstract]. J Bone Miner Res 2006；21 Suppl 1：S75.
18) Kaplan FS, Glaser DL, Shore EM, et al. Hematopoietic stem-cell contribution to ectopic skeletogenesis. J Bone Joint Surg Am 2007；89：347.

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話　題

進行性骨化性線維異形成症（FOP）の病因と病態*

片桐岳信**

Key Words：fibrodysplasia ossificans progressiva（FOP），bone morphogenetic protein（BMP），myoblast，muscle regeneration，receptor

はじめに

進行性骨化性線維異形成症（fibrodysplasia ossificans progressiva；FOP）は，以前には進行性化骨筋炎（myositis ossificans progressiva；MOP）とも呼ばれた疾患で，全身の骨格筋を中心に靱帯や腱における異所性骨形成を特徴とする疾患である1)（図1）．FOP患者は成長に伴って多くの関節が癒合するため，進行に伴い可動域が極度に小さくなり自立的な運動が困難となる．

現時点で，FOPの異所性骨化を抑制できるような有効な治療法は確立されていない．FOPは，厚生労働省が示す難治性疾患克服研究事業対象疾患（難病）の4要件（希少性，単因不明，治療法未確

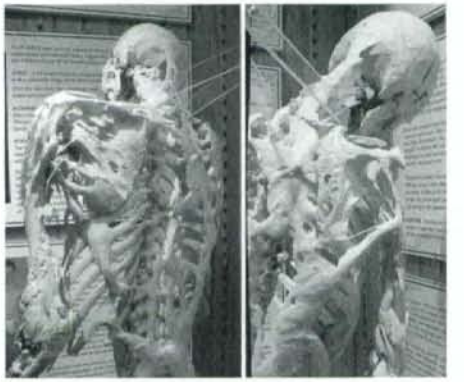

図1　FOPにおける異所性骨形成
米国ペンシルバニア州フィラデルフィアのMutter Museumに展示されているFOPの骨格標本．許可をとって撮影した．

* Fibrodysplasia ossificans progressiva.

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立，生活面で長期にわたる支障）をすべて満たすことから，2007年3月の特定疾患対策懇談会で色素性乾皮症(XP)とともに新しい難病に追加認定された．これにより，FOPはわが国の123の難病の一つとして，2007年4月から厚生労働省の研究班によって治療法の確立に向けた研究が開始された．

一方，これまでFOPは，症例数が極度に少ないこともあり医療関係者にも知られていない場合があり，このことがFOPの診断や治療に大きな影響を与えてきた．難病に認定されたFOPの理解を深めることは，治療法が確立されていない現状において，少しでも進行を遅延させることに繋がる可能性がある．本稿では，最近の研究成果を交えながらFOPを概説する．

FOPの病態

1．発症頻度と遺伝

FOPの発症率は，1982年に発表されたイギリスの研究で約200万人に1人と推定され，明確な人種差や地域差はなく世界的にも同程度と考えられている[1]．1家系に複数のFOP患者が確認された家族性FOPとして，一卵性双生児の双方に発症した例や，父親から女児を含む複数の子供に遺伝した症例が海外で7例確認されているが，FOPのほとんどは孤発性で両親や兄弟を含む血縁者に異常は認められない[2-4]．FOPの遺伝様式は，常染色体優性遺伝であることが判明した．

日本の人口を約1億2,000万人とすると，世界的な発症率から約60人のFOP患者が存在すると推定される．2008年6月に報告された厚労省FOP研究班の医療機関を対象としたアンケート調査結果でも，約60人のFOP患者が確認された[5]．現在，国内で生存が確認されているFOPの症例はすべて孤発性である．唯一，FOPの診断が確定した患者の一卵性双生児の姉妹に，FOPで特徴的な母趾の変形が認められたとする1例があるが，姉妹がすでに死亡しているため診断がついていない．

2．異所性骨化の進行

FOPでは，さまざまな骨格筋，靭帯，腱に異所性骨形成を生じる一方，心筋，横隔膜，舌に骨化を認めた症例はない．FOP患者でも，出生時には明らかな異所性骨形成は認めない．しかし，4～5歳までに約50%の症例に異所性骨化が起こり，15歳までにほぼ全症例で骨化が認められるという[6]．FOPにおける異所性骨形成は，体幹から四肢へ，頭部側から尾部側へ，背部から腹部へ，筋位から遠位へ進行する特徴がある[6]．FOPの異所性骨形成は成長期に著しく進行し，20～30%の患者に難聴が認められる．

FOPの異所性骨形成は，成長とともに進行するばかりでなく，外傷などによって急激に進行する．このため，手術，バイオプシー，筋組織内への注射など侵襲的な医療行為は禁忌とされている．しかし，世界的なFOP患者を対象としたアンケート調査によると，約67%はなんらかの侵襲的医療行為の経験があり，その結果，そのうち約35～60%は骨化などの後遺症が誘発された(表1)[6]．

表1　FOPの侵襲的医療行為と後遺症

診断・治療		後遺症
バイオプシー	67%	35%
手術	35%	60%

（文献[6]より引用改変）

3．外反母趾とフレア・アップ

FOPの発症前の外見的特徴として，ほぼすべての症例で出生時から左右対称性に外反母趾様の変形が認められることが知られている[1,6]．さらに，手の小指や親指にも短縮や変形が認められる場合もある．これは，後述するように，FOPがbone morphogenetic protein(BMP)受容体の変異によるためで，BMPが胎児の発生段階で四肢の形成に重要なこととよく一致する．

2～5歳前後になると，筋組織内での骨形成に先立ち，頭，頚，背中に痛みを伴うフレア・アップと呼ばれる腫脹が数週間持続して出現する[1,6]．その後，腫瘤の消失に伴い異所性骨形成が進行することから，局所的なフレア・アップの反応が骨形成を誘発している可能性がある．FOPにおける外傷性の局所的骨形成の場合にも，骨化に先立ってフレア・アップが起こることが知られている．FOPのフレア・アップは，インフルエンザウイルスの感染によっても起こる[7]．FOPではバイオプシーが禁忌のためフレア・アップに関する組織学的情報が乏しいが，比較的初期の腫瘤は炎症物質や線維芽細胞で満たされている．このため，バイオプシーを行った場合でも，FOPは癌と誤診されるケースが多い(表2)[6]．FOPのフレア・アップが起こった部位には，ステージによらず多数の肥満細胞を認めたという報告がある[8]．

表2　FOPの診断における特徴

診断までの年数	4.1年
診断までの医師数	6人
初診での診断率	13%
誤診される疾患	癌(32%)

（文献[6]より引用改変）

FOPの発症機序

1．BMPシグナル(図2)

FOPと同様に，筋組織内で異所性骨化を誘導できるサイトカインとしてBMPが知られている[9]．BMPは，transforming growth factor-β(TGF-β)ファミリーに分類される一次構造の類似した因子で，15種類以上のBMPがBMP-2/4, BMP-5/6/7/8, BMP-9/10, BMP-12/13/14, BMP-3などのサブファミリーに分けられる[9]．

BMPの骨誘導シグナルは，標的細胞のⅠ型とⅡ型受容体を介して細胞内に伝達される(図2)[9]．どちらも膜貫通型セリン・スレオニンキナーゼ受容体であり，BMPを結合するとⅡ型受容体がⅠ型受容体をリン酸化によって活性化する．さらに，活性化されたⅠ型受容体が細胞質で転写因子Smad1/5/8をリン酸化する．Smad1/5/8は，Smad4と複合体を形成して核内へ移行し，標的遺伝子上の特異的な塩基配列を認識して結合し転写を制御すると考えられている．

2．責任遺伝子

FOPとBMPの骨形成が類似していることから，FOPはBMPシグナルの異常が指摘されていた．1996年には，FOP患者のリンパ球ではBMP-4の発現量が増加していることが報告された[10]．さらに，BMP自身だけでなくほかのBMP制御因子の変異との関連も指摘された．この1例として，細胞外でBMPに結合してBMP受容体への結合を阻害するnogginをコードする遺伝子に，FOP患者における欠失や点変異が報告されている[11]．しかし，nogginの欠失が同定された同一患者の遺伝子を解析しても，変異を追試できないとの報告があ

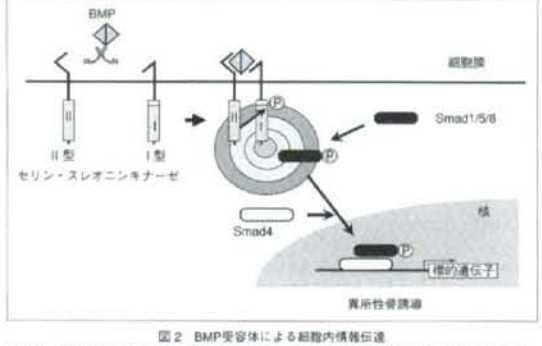

図2　BMP受容体による細胞内情報伝達
BMPは，標的細胞表面の2種類の膜貫通型セリン・スレオニン受容体(Ⅰ型とⅡ型)に結合する．Ⅱ型受容体がⅠ型受容体をリン酸化によって活性化し，Ⅰ型受容体がさらに下流の転写因子Smad1/5/8をリン酸化反応によって活性化する．Smad1/5/8は，Smad4と複合体を形成して核へ移行し，標的遺伝子の転写を制御する．（筆者作成）

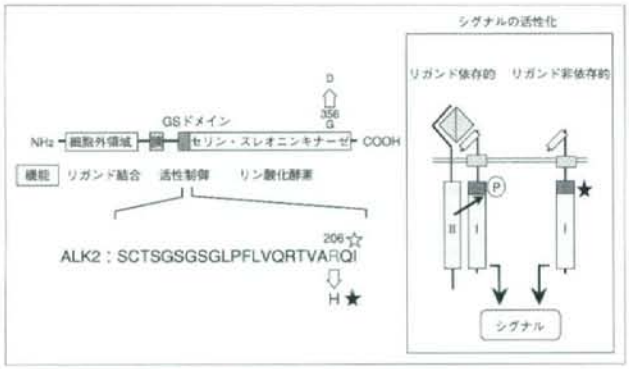

図3　ALK2受容体のアミノ酸置換による活性化とFOPにおける変異
BMPやTGF-βの受容体は，GSドメインの保存されたTYSアミノ酸を置換するとリガンド非依存的に活性化されることが知られていた．FOPで同定されたALK2(R206H)変異はこの隣のアミノ酸であり，受容体を構成的に活性化することが確認された．最近，国内のFOP症例で，R206Hではなく ALK2(G356D)変異が同定された．（筆者作成）

る[3]．FOPの遺伝学的連鎖解析から，*Smad1*遺伝子を含む4番染色体q27-31領域との相関も指摘された[13]．

2006年，家族性FOPの連鎖解析から，FOPの責任遺伝子として2番染色体q23-24に存在する*ACVR1*遺伝子が同定された[14]．*ACVR1*はBMPのI型受容体の一つであるALK2をコードする遺伝子で，FOP患者では617番目の塩基がGだけでなくAのヘテロ接合体であることが判明した．この変異は，家族性FOPのみならず，海外および国内の孤発性FOPにも共通して認められることが確認されている[14-16]．ごく最近，同じ*ACVR1*遺伝子の1,067番にG>Aの新しいタイプのFOP患者が見出された[17]．これらの発見は，ACVR1の変異がFOPの発症と密接に関係することを示す．

3．BMPシグナルの亢進

FOPで見出された*ALK2*の2種類の変異は，どちらもアミノ酸変異をひき起こす．*ACVR1*遺伝子の617G>Aは206番のアルギニンをヒスチジン残基(R206H)へ，1,067G>Aは356番のグリシンをアスパラギン酸(G356D)へ変異させる(図3)[14-17]．

BMPを含むTGF-βファミリーのI型受容体は，細胞内のキナーゼドメインの直上にグリシンとセリン残基に富む“GSドメイン”と呼ばれる構造をもつ．このGSドメインはII型受容体によってリン酸化される領域で，I型受容体の活性制御領域として機能する．このGSドメインの保存されたアミノ酸を酸性アミノ酸に置換すると，リガンドやII型受容体に非依存的にシグナルが活性化されることが示されている(図3)[18]．とくに大部分のFOPで同定されたALK2(R206H)は，この構成的活性化に関与する隣のアミノ酸残基であることから，FOPにおけるALK2受容体の活性化が示唆された．

ALK2(R206H)受容体の活性を培養細胞を用いて野生型と比較すると，Smad1/5/8のリン酸化，標的遺伝子の活性化，筋分化の抑制活性などから，構成的活性型BMP受容体であることが確認された[19,20]．この変異受容体を発現した細胞は，BMP刺激に高感受性になるらしい．ただし，このALK2(R206H)受容体の活性は比較的弱く，骨芽細胞の分化誘導にはSmad1やSmad5が同時に発現することが必要であった．マウスの筋再生モデルでは，ALK2の発現量は変化しないが，SmadとSmad5の量が増加した[19]．FOPでは，遺伝的に活性化されたALK2と，筋再生に伴って増加するSmad1やSmad5との協調的な作用で骨化が進行する可能性がある(図4)．

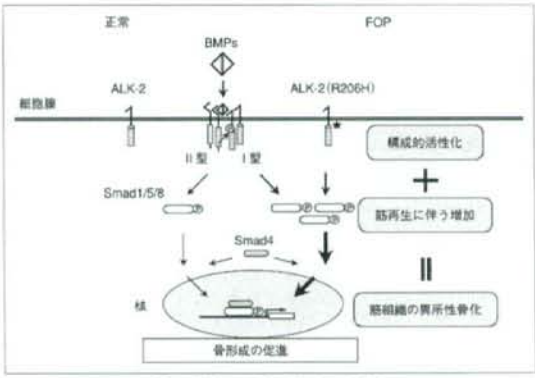

図4　FOPにおけるBMPシグナルの変異
野生型のALK2は，BMPと結合したときのみSmad1/5/8を介して骨形成を誘導する．一方，FOPのALK2(R206H)受容体は，GSドメインの変異によりBMPが結合しなくとも構成的にSmad1/5/8を活性化する．ALK2を阻害する分子は，FOPの治療に応用できる可能性がある．（筆者作成）

FOPの診断と治療

1．診断の現状と今後(表2)

従来，FOPの診断は異所性骨化に基づいたものであったため，発症から確定診断までに平均4年以上，6人以上の医師を要していた[2]．この間にバイオプシーや腫瘤の除去手術などが行われた結果，骨化を誘発した例が多数報告されている[21]．上述のように，FOPが*ALK2*の単一遺伝子の変異に基づくと予想されることから，遺伝子診断の有効性が考えられる．また，出生時からの外反母趾様の変形は，これまで見過ごされてきた重要な外見上の特徴であることを再認識する必要がある．実際，埼玉医科大学FOP診療・研究プロジェクトにおいて，外反母趾が認められ，かつフレア・アップのある小児において，*ALK2*の遺伝子診断によって明らかな異所性骨化の発症前にFOPが確認された症例が複数ある．

2．免疫抑制剤

現在，FOPに対しては，主にフレア・アップの抑制を目的とした短期のステロイドの大量投与や，異所性骨化の抑制を目的としたビスフォスフォネートの投与が行われている[2]．しかし，FOPの異所性骨化を抑制できるような治療法は確立されていない．

最近，FOP患者で骨髄不良性貧血に伴う骨髄移植を行った症例が報告された．この症例では，免疫抑制剤が投与されていた14年間は，ほとんど異所性骨化が起こらなかったという[22]．しかし，免疫抑制剤の投与中止後には異所性骨化が再発したことから，骨髄移植はFOPを治療できないことも明らかとなった．今後，再現性を含めて，免疫抑制剤による異所性骨化の抑制機序の解明が注目される．

3．BMP受容体阻害剤

FOPの発症原因としてALK2受容体の機能亢進が明らかとなりつつあることから，受容体の阻

害剤がFOP治療に応用できる可能性が示唆される．最近発見されたBMP受容体に対する低分子阻害剤(dorsomorphin)は[20]，培養細胞レベルでALK2(R206H)の活性を阻害することが確認された[20]．さらに特異性が高く，低毒性の低分子化合物が開発されることが期待される．

おわりに

以前から，bone morphogenetic protein(BMP)活性との関連が示唆されてきた進行性骨化性線維異形成症(fibrodysplasia ossificans progressiva；FOP)が，bone morphogenetic protein(BMP)受容体の一つである*ALK2*遺伝子の変異に基づくことが明らかとなってきた[21]．これにより，FOPに有効な治療法を開発するための方向性が示されている．治療法が確立されていない現時点では，遺伝子診断によってできるだけ早期にFOPを確定診断し，その後の生活様式などを検討して骨化を遅らせることも大切である．

FOPは，その特性から患者の組織を解析することが困難なことが研究上の大きな障害であった．FOPの発症原因解明や治療法確立のために，変異*ALK2*遺伝子を用いた病態モデルの早急な確立が望まれる．

謝辞：本稿は，埼玉医科大学ゲノム医学研究センター病態生理部門，埼玉医科大学FOP診療・研究プロジェクト，厚生労働省難治性疾患克服研究事業「骨化筋炎骨化症に関する調査研究」班および「特定疾患患者の生活の質(quality of life；QOL)の向上に関する研究」班から多大な協力を得たものであり，ここに感謝の意を表する．また，本研究の一部は，文部科学省学術研究高度化推進事業・学術フロンティア，埼玉医科大学学内グラント，日本学術振興会科学研究補助金，厚生労働科学研究補助金，三共生命科学振興財団，川野正登記念(財)川野小児医学奨学財団，ノボノルディスク成長・発達賞の助成を受けた．

文　献

1) Kaplan FS, Shen Q, Lounev V, et al. Skeletal metamorphosis in fibrodysplasia ossificans progressiva. J Bone Miner Metab. In press 2008.
2) Connor J, Evans DA. Genetic aspects of fibrodysplasia ossificans progressiva. J Med Genet 1982 ; 19 : 35.
3) Eaton WL, Conkling WS, Daeschner CW. Early myositis ossificans progressiva occurring in homozygotic twins ; a clinical and pathologic study. J Pediatr 1957 ; 50 : 591.
4) Kaplan FS, McCluskey W, Hahn G, et al. Genetic transmission of fibrodysplasia ossificans progressiva. Report of a family. J Bone Joint Surg Am 1993 ; 75 : 1214.
5) 芳賀信彦，川端秀彦，鬼頭浩史，ほか．日本における進行性骨化性線維異形成症患者の診療状況に関する研究．In：厚生労働省科学研究費補助金難治性疾患克服研究事業「骨化線筋骨化症に関する調査研究」平成19年度総括・分担研究報告書．2008. p. 165.
6) Kitterman JA, Kantanie S, Rocke DM, et al. Iatrogenic harm caused by diagnostic errors in fibrodysplasia ossificans progressiva. Pediatrics 2005 ; 116 : e654.
7) Scarlett RF, Rocke DM, Kantanie S, et al. Influenza-like viral illnesses and flare-ups of fibrodysplasia ossificans progressiva. Clin Orthop Relat Res 2004 ; 423 : 275.
8) Gannon FH, Glaser D, CaronR, et al. Mast cell involvement in fibrodysplasia ossificans progressiva. Hum Pathol 2001 ; 32 : 842.
9) Katagiri T, Suda T, Miyazono K. The Bone Morphogenetic Proteins. In The TGF-β Superfamily Monograph. In : Miyazono K, Derynck R, editors. New York : Cold Spring Harbor Press ; 2008. p. 121.
10) Shafritz AB, Shore EM, Gannon FH, et al. Overexpression of an osteogenic morphogen in fibrodysplasia ossificans progressiva. N Engl J Med 1996 ; 335 : 555.
11) Lucotte G, Sémonin O, Lutz P. A *de novo* heterozygous deletion of 42 base-pairs in the noggin gene of a fibrodysplasia ossificans progressiva patient. Clin Genet 1999 ; 59 : 469.
12) Xu MQ, Feldman G, Le Merrer M, et al. Linkage exclusion and mutational analysis of the noggin gene in patients with fibrodysplasia ossificans progressiva (FOP). Clin Genet 2000 ; 58 : 291.
13) Feldman G, Li M, Martin S, et al. Fibrodysplasia ossificans progressiva, a heritable disorder of severe heterotopic ossification, maps to human chromosome 4q27-31. Am J Hum Genet 2000 ; 66 : 128.
14) Shore EM, Xu M, Feldman GJ, et al. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet 2006 ; 38 : 525.
15) 片桐岳信，福田　亨，神田理和，ほか．Fibrodysplasia Ossificans Progressiva(FOP)におけるBMP受容体ALK2の変異とその機能解析．日整会誌 2006 ; 80 : 710.
16) Nakajima M, Haga N, Takikawa K, et al. The ACVR1 617G>A mutation is also recurrent in three Japanese patients with fibrodysplasia ossificans progressiva. J Hum Genet 2007 ; 52 : 473.
17) Furuya H, Ikezoe K, Wang L, et al. A unique case of fibrodysplasia ossificans progressiva with an ACVR1 mutation, G356D, other than the common mutation (R206H). Am J Med Genet A 2008 ; 146A : 459.
18) Wieser R, Wrana JL, Massagué J. GS domain mutations that constitutively activate TβR-I, the downstream signaling component in the TGF-β receptor complex. EMBO J 1995 ; 14 : 2199.
19) Fukuda T, Kohda M, Kanomata K, et al. A constitutively activated BMP receptor, ALK2, induces heterotopic bone formation in patients with fibrodysplasia ossificans progressiva (FOP) [abstract]. J Bone Miner Res 2007 ; suppl 1 : S10.
20) Shen Q, Xu M, Little SC, et al. Activation of BMP signaling by the FOP ACVR1 R206H mutaion [abstract]. J Bone Miner Res 2007 ; suppl 1 : S43.
21) Kaplan FS, Glaser DL, Shore EM, et al. Hematopoietic stem-cell contribution to ectopic skeletogenesis. J Bone Joint Surg Am 2007 ; 89 : 347.
22) Yu PB, Hong CC, Sachidanandan C, et al. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 2008 ; 4 : 33.
23) Fukuda T, Kohda M, Kanomata K, et al. Constitutively activated ALK2 and increased smad1/5 cooperatively induce BMP signaling in fibrodysplasia ossificans progressiva. J Biol Chem 2008 Aug 6 [Epub ahead of print].

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ORIGINAL ARTICLE

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Cyclic AMP enhances Smad-mediated BMP signaling through PKA-CREB pathway

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Abstract We present experimental results indicating involvement of cyclic AMP (cAMP)-mediated signaling in bone morphogenetic protein (BMP)-induced osteoblastic gene expression at the transcriptional level by luciferase activity assay in C2C12 cells using the promoter sequence of the *Id1* gene, an early-response gene to BMPs, which contains both a BMP-responsive element (BRE) and a cAMP-response element (CRE). In cells transfected with luciferase gene driven by wild-type *Id1* promoter, treatment with BMP-4 increased luciferase expression, which was further enhanced by the addition of dibutyryl cAMP (dbcAMP). This dbcAMP-enhanced luciferase expression was significantly suppressed when the CRE site in the *Id1* promoter was replaced by mutated CRE or endogenous CRE-binding protein (CREB) was knocked down by transfection of CREB RNAi. Pretreatment of cells with protein kinase A (PKA) inhibitor, H89, also dramatically reduced dbcAMP-enhanced luciferase expression. Immunoprecipitation assay showed phosphorylated-Smad1/5/8, phosphorylated-CREB, and CREB-binding protein (CBP) formed the transcriptional complex. These data indicate that cAMP-PKA/CREB/CRE signaling potentially enhances BMP-induced transcription through the BRE in the promoter of the BMP-responsive gene through a PKA-mediated pathway.

Key words bone morphogenetic protein (BMP) · cyclic adenosine 3′,5′-monophosphate (cAMP) · protein kinase A (PKA) · CRE-binding protein (CREB) · cyclic AMP-response element (CRE)

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Introduction

Bone morphogenetic proteins (BMPs) are a family of multifunctional growth factors belonging to the transforming growth factor (TGF)-β superfamily. Some BMP members exhibit a potent capacity to induce differentiation of mesenchymal cells into osteo- or chondrogenic lineage cells. The specific biological capacity of BMPs is mediated by two receptors (BMPRI and BMPRII) with serine-threonine kinase activity that are anchored to the cytoplasmic membrane. The BMPR/BMP complex phosphorylates BMP-specific intracellular signaling molecules, regulatory Smad molecules (Smad1, Smad5, and Smad8) that further bind to common Smad (Smad4) to form a heterotrimer transcriptional complex, which translocates into the nucleus and regulates gene transcription by binding to Smad-specific binding sites on the promoters of target genes to regulate transcription [1] and thus express osteo- or chondrogenic phenotypes.

The anabolic action of BMP in osteogenic differentiation is reportedly enhanced by the addition of a nonselective phosphodiesterase (PDE) inhibitor (pentoxifylline), a cyclic adenosine 3′,5′-monophosphate (cAMP)-specific PDE-4 inhibitor (rolipram), and a prostaglandin E_2 EP4 receptor selective agonist (ONO-4819), respectively, for both in vivo and in vitro conditions. The anabolic effects of these agents on osteogenic differentiation are seen exclusively in the presence of BMP [2–7]. As the common intracellular event caused by those agents is the elevation of intracellular cAMP level, we hypothesized that cAMP would enhance osteoblastic differentiation through modulating BMP signaling for osteoblastic gene expression, probably via the cAMP-response element (CRE) in the promoter sequences of target genes through the protein kinase A (PKA)/CRE-binding protein (CREB) pathway. To substantiate this hypothesis, modification of BMP-induced transcription by the cAMP-mediated signaling pathway was investigated in C2C12, a muscle-derived mesenchymal cell line expressing BMPRI and BMPRII [8], using the promoter of the *Id1* gene, which is an early-response gene for BMPs and has both a BMP-responsive element (BRE) and a CRE in its promoter region [9–11].

Materials and methods

Reagents

A cell membrane permeable analogue of cAMP, dibutyryl cAMP (dbcAMP), was purchased from Sigma (St. Louis, MO, USA) and used to prepare 100 mM stock solution in sterile water. Forskolin (Sigma) was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). An inhibitor specific to PKA, H89 (D. Western Therapeutics Institute, Nagoya, Japan) was prepared as a 10 mM stock solution in DMSO.

Conditioned medium was obtained from culture media of Chinese hamster ovary (CHO) cells transfected with murine BMP-4 (mBMP-4) cDNA and was used as a source of mouse BMP-4 as previously described [12]. Based on capacity for alkaline phosphatase (ALP) induction in osteoblastic cells (ST2 cells), titer of BMP-4 in the media corresponded to approximately 100 ng/ml recombinant human BMP-2 (generously provided by Yamanouchi Pharmaceutical, Tokyo, Japan) (data not shown).

Cell culture

C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in α-minimal essential medium (α-MEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) for growth or 2.5% FBS for experiments and antibiotic/antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Sigma) at 37°C in 5% CO_2 humidified air. Upon reaching confluence, cells were used for the following experiments.

Construction of reporter plasmids

To determine the effects of cAMP on BMP-induced transcriptional activity in C2C12 cells, we constructed a series of luciferase reporter plasmids with a mutation in either the BRE or CRE, or in both the BRE and CRE in the *Id1* promoter sequence. The wild-type human *Id1* gene promoter sequence region (−985 bp to +94 bp) contains both the BRE and CRE, and those elements locate closely in the sequence (−985 bp to −957 bp, −928 bp to −921 bp, respectively) [9–11]. *Id1* promoter sequences with mutated BRE (*mut* BRE) were prepared as previously described [9]. To generate mutation in the CRE site (TGACGTCA), in wild-type and *mut* BRE promoters, PCR amplification was performed by Pyrobest DNA polymerase (Takara Bio, Otsu, Japan) using the sense and antisense primers described below:

sense:
5′-GTCCGGGTTTTATGAATGGGGTCAGTCA
CAGGCCTGGCGTCTAACGGT-3′;

and antisense:
5′-ACCGTTAGACGCCAGGCCTGTGACTGACCCCA
TTCATAAAACCCGGACGGACTG-3′.

PCR products were generated using a Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the supplied instructions with slight modifications, and plasmids were digested with Dpn I (Stratagene).

Transfection and relative luciferase activity assay

Cells were seeded at a density of 1×10^5 cells/well in 96-well plates with growth media. At near confluence, cells were transfected with the reporter plasmid constructs with or without mutated BRE and/or CRE sequences using Lipofectamine 2000 according to instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). At 5 h after transfection, medium was changed with fresh media, then cells were treated with either BMP-4 (5% vol/vol), or dbcAMP (1 mM)/forskolin (10^{-5} M), or both for 24 h, followed by luciferase activity assay. In a separate treatment, cells were cotransfected with a dominant-negative inhibitor of CREB, termed the A-CREB plasmid (kindly provided by Dr. Charles Vinson, Laboratory of Metabolism NCI, NIH, USA) [13] using Lipofectamine 2000 (Invitrogen).

As an internal control, each construct was used in combination with a control luciferase vector (pRL) to calculate transfection efficiency. Luciferase activities in cell extracts were determined using a Dual-Glo Luciferase Reporter Assay System (Promega, Madison, WI, USA), as described previously [9].

RNAi design and transfection

Double-stranded RNAi Stealth select oligos (MSS236241) for CREB were purchased from Invitrogen. Cells were seeded at a density of 1×10^5 cells/well in 96-well plates with growth media, and transfections were performed using Lipofectamine 2000 according to instructions of the manufacturer (Invitrogen). For negative controls, BLOCK-iT Fluorescent Oligo (Invitrogen) was transfected as described above.

Determination of ALP activity

For the ALP assay, C2C12 cells were seeded at a density of 5×10^4 cells/well in 24-well plates ($n = 6$/group).

On achieving confluence, cells were treated with or without BMP-4 conditioned medium (5% vol/vol) and dbcAMP (1 mM), and incubation was continued for additional 6 days. The medium was removed at this point, and cells were then washed three times with saline and lysed in 200 μl 0.2% Triton-X (Sigma) with saline. Plates were then sonicated in a defined sequence at 4°C using a Bioruptor sonicator (Cosmo Bio, Tokyo, Japan). After centrifugation,

supernatants were collected and assayed for ALP activity as described previously [7]. Activity was normalized by protein determined with the protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to Lowry's method. Independent experiments were performed in triplicate.

Coimmunoprecipitation experiments

After concurrent treatment of C2C12 cells with BMP-4 conditioned medium (5% vol/vol) and dbcAMP (1 mM) for 1 h, cells were washed twice with ice-cold PBS, scraped from dishes with a plastic scraper, and suspended in 1 ml of ice-cold Radio-Immunoprecipitation Assay (RIPA) buffer containing protease inhibitors (1 mM PMSF and 50 μg/ml protease inhibitor cocktail; Sigma). Cells were lysed by sonication, and lysed fragments were pelleted by centrifugation for 10 min at 13,000 rpm at 4°C. A 50% slurry of Protein A Sepharose 4 Fast Flow beads in RIPA buffer was prepared using the IP Starter Pack (Amersham Biosciences, Piscataway, NJ, USA) as described in the instructions supplied by the manufacturer. Supernatants from pelleted cell lysates (500 μl) were mixed with antibodies against phosphorylated-CREB (p-CREB; Cell Signaling Technology, Danvers, MA, USA), CBP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or phosphorylated-Smad1/5/8 (p-Smad1/5/8; Cell Signaling Technology) overnight at 4°C. To collect antibody–protein complexes, 50 μl 50% slurry of Protein A Sepharose was added per 500 μl cell lysate and mixed for 1 h. IP beads were then collected by centrifuging at 1000 rpm at 4°C for 2 min. Supernatant was discarded, and the beads were washed twice with 1 ml RIPA buffer and once with 50 mM ethylenediaminetetraacetic acid (EDTA). Thereafter, IP beads were processed for sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) analysis. Proteins in the electrophoresed gel were blotted onto the Immobilon-P Transfer Membrane (Millipore, Billerica, MA, USA) and detected by Western blotting with use of antibodies against the proteins of interest, i.e., p-Smad1/5/8 and CBP. Proteins bound to respective antibodies were visualized using an ECL+ detection system (Amersham Biosciences).

Statistical analysis

Data are expressed as mean ± standard deviation (SD) for each group. Differences between treatment groups were analyzed using Fisher's protected least significant difference (PLSD) test. Values of $P < 0.05$ were considered statistically significant.

Results

Cyclic AMP enhances BMP-induced ALP induction

ALP was consistently induced in C2C12 cells by BMP, as reported previously [14]. ALP induction by BMP-4 was significantly enhanced by dbcAMP, whereas addition of dbcAMP alone showed no significant elevation of ALP in C2C12 cells (Fig. 1). From this result, it was confirmed that dbcAMP enhanced BMP-induced-osteoblastic differentiation in C2C12 cells.

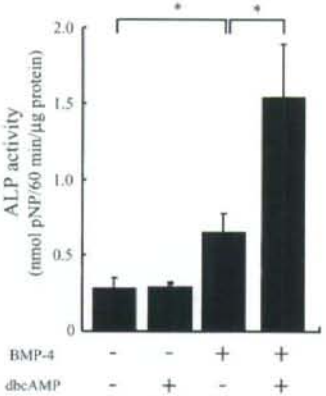

Fig. 1. Cyclic adenosine 3',5'-monophosphate (cyclic AMP) significantly intensified bone morphogenetic protein (BMP)-4-induced alkaline phosphatase (ALP) induction in C2C12 cells. C2C12 cells were treated with BMP-4-conditioned medium (5% vol/vol) and dibutyryl cAMP (dbcAMP) (1 mM). After 6 days, ALP activities were measured. Results represent mean ± SD for three cultures. Experiments were performed three times with similar results. *$P < 0.001$

Cyclic AMP accelerates BMP signaling by enhancing transcriptional activity of the BRE through the CRE mediated by the PKA pathway

Relative luciferase activity (RLA) in C2C12 cells transfected with reporter plasmids containing wild-type *Id1* promoter was increased by BMP-4 treatment and further enhanced by the addition of dbcAMP (Fig. 2A). RLA level in cells transfected with reporter plasmids containing the mutated CRE in *Id1* promoter was increased by BMP-4 treatment, but no further elevation of RLA was detected by addition of dbcAMP (Fig. 2B). This result indicates that cAMP accelerates BMP signaling by enhancing transcriptional activity of the BRE through the CRE. Cells transfected with the reporter construct containing the mutated BRE did not show luciferase induction with either wild-type or mutated CRE (Fig. 2C,D). Control cells transfected with pGL3 mock vector had no effect on RLA by treatment with BMP-4 or dbcAMP (data not shown).

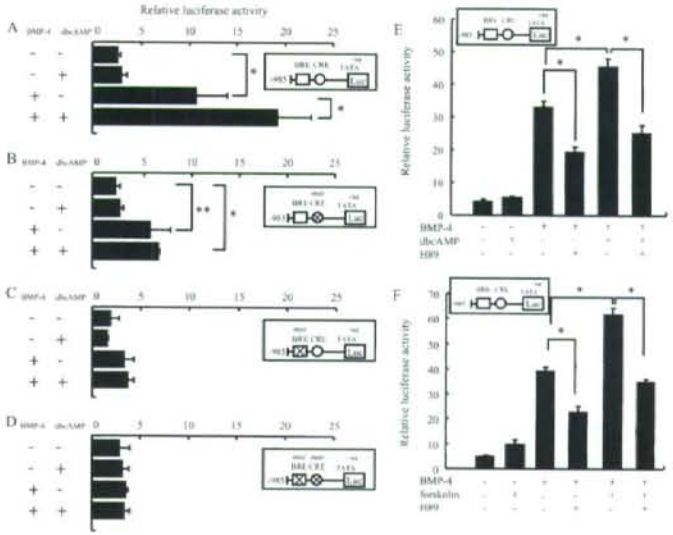

Fig. 2. Cyclic AMP enhances BMP-induced transcriptional activity of the BMP-responsive element (BRE) through the cyclic AMP-response element (CRE) in the *Id1* gene promoter. C2C12 cells were transfected with a series of luciferase reporter plasmids with a mutation in either the BRE or CRE in the *Id1* promoter sequence and treated with or without BMP-4 (5% vol/vol) or dbcAMP (1 mM). Relative luciferase activity (RLA) was determined in cells transfected with *Id1* reporter plasmids containing wild-type BRE and wild-type CRE (**A**), wild-type BRE and mutated CRE (**B**), mutated BRE and wild-type CRE (**C**), or mutated BRE and mutated CRE (**D**). **E**, **F** RLA in cells transfected with *Id1* reporter plasmids containing both wild-type BRE and wild-type CRE and pretreated either with 2 μM H89, a specific protein kinase A (PKA) inhibitor, or vehicle for 1 h and then treated with or without BMP-4 (5% vol/vol) or dbcAMP (1 mM)/forskolin (10^{-5} M). Results represent mean ± SD for three cultures. Experiments were performed three times with similar results. *$P < 0.001$, **$P < 0.01$

To examine the role of PKA pathways in cAMP-mediated enhancement of BMP-induced transcriptional activity in C2C12 cells, H89 was used as a PKA inhibitor. Enhanced elevation of BMP-4-induced RLA by dbcAMP in C2C12 cells transfected with reporter plasmids containing wild-type *Id1* promoter was significantly decreased by H89 treatment (Fig. 2E). These effects were fully mimicked by forskolin, a selective agonist of cAMP production (stimulator of adenylate cyclase) (Fig. 2F).

These observations strongly suggest that PKA is involved in the enhancement of BMP-induced transcriptional activity by cAMP.

Endogenous CREB is involved in Smad-mediated BMP signaling and CRE-mediated enhancement

To examine the role of CREB in enhancing BMP signaling through the CRE downstream of PKA, CREB expression was downregulated in C2C12 cells by RNA interference (RNAi). We observed CREB RNAi resulted in 70% decreases of the endogenous CREB protein in Western blotting (data not shown).

Transfection of CREB RNAi and wild-type *Id1* promoter construct, and treatment with BMP-4 and dbcAMP, then indicated that suppression of endogenous CREB

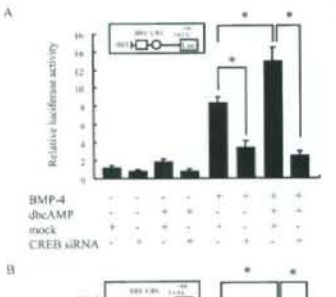

Fig. 3. Downregulation of endogenous CREB suppressed enhancement of BMP-induced transcriptional activity by cAMP. **A** RLA of wild-type *Id1* promoter in cells transfected with either CREB-specific RNAi or control siRNA and treated with or without BMP-4 (5% vol/vol) or dbcAMP (1 mM). **B** RLA in cells cotransfected with wild-type *Id1* and A-CREB plasmid, or CMV500 empty vector, and treated with or without BMP-4 (5% vol/vol) or dbcAMP (1 mM). Results represent mean ± SD for three cultures. Experiments were performed three times with similar results. *$P < 0.001$

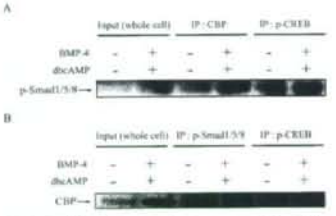

Fig. 4. p-CREB binds directly or indirectly to p-Smad1/5/8 and CBP. **A, B** C2C12 cells were treated with BMP-4 (5% vol/vol) and dbcAMP (1 mM) for 60 min. Coimmunoprecipitation and Western blotting were performed using anti-p-Smad1/5/8 antibodies and anti-p-CREB antibodies, or anti-p-Smad1/5/8 antibodies and anti-CBP antibodies, respectively. A 10% volume of each sample was loaded as a control.

expression by CREB RNAi resulted in decreased cAMP-dependent enhancement of Smad-mediated transcription by BMP-4 (Fig. 3A). A similar effect was shown when cells were co-transfected with the reporter plasmid containing wild-type *Id1* promoter and A-CREB, a competitive inhibitor of CREB (Fig. 3B).

Formation of transcriptional factor complex involving p-CREB, CBP, and p-Smad1/5/8

Coimmunoprecipitation experiments revealed that proteins immunoprecipitated with anti-p-CREB antibodies recovered from lysates of cells treated with BMP-4 and dbcAMP included p-Smad1/5/8 and CBP. In addition, proteins immunoprecipitated with anti-CBP antibodies included p-Smad1/5/8 and proteins immunoprecipitated with anti-p-Smad1/5/8 antibodies included CBP on Western blot analyses (Fig. 4A,B). These results suggest that p-Smad1/5/8 activated by BMP stimulation, p-CREB mainly induced by cAMP/PKA signaling, and CBP known as histone acetyl transferase form the transcriptional complex by direct or indirect binding, and support that a part of the cross-talk at the transcriptional level between BMP signaling and cAMP signaling is elucidated.

Discussion

The present experimental study demonstrated the mechanism of enhancement for Smad-mediated BMP signaling by cAMP/PKA/CREB/CRE signaling at the transcriptional level.

Previous groups, including ours, have reported that pentoxifylline (a nonselective PDE inhibitor), rolipram (inhibitor selective to PDE4), ONO-4819 (a prostaglandin E_2 EP4 receptor selective agonist compound), and parathyroid hormone (PTH) can promote BMP-induced osteoblastic differentiation [2–7]. The common intracellular event caused by these agents is the transient elevation of cAMP levels. However, no exact explanation for the enhanced BMP-mediated osteogenic differentiation by those agents or by elevated intracellular cAMP levels has been presented. The present study investigated interactions between cAMP and BMP signaling at the transcriptional level using the promoter of the *Id1* gene, an early-response gene to BMPs, containing both the BRE and CRE [9–11] and which might thus offer a suitable tool for this study.

The results indicate that the anabolic effect of cAMP on BMP-induced osteoblastic differentiation is mediated by enhancing transcriptional activity of the BRE through the CRE, in which the PKA signaling pathway is involved, and BRE/p-Smads and CRE/p-CREB complexes appear to be linked by a transcriptional cofactor complex including CBP.

With regard to signaling pathway following elevation of cAMP level, the canonical PKA/CREB/CRE signaling pathway would represent a major pathway, as significant suppression of enhancing effects on BMP signaling by PKA inhibitor and CREB inhibitor was seen.

Downstream of the PKA pathway, we analyzed the role of CREB using CREB RNAi and dominant-negative inhibitor of the CREB plasmid. CREB RNAi and A-CREB significantly decreased the RLA enhanced by dbcAMP. Interestingly, inhibition of BMP signaling was observed when endogenous CREB was knocked down using RNAi (see Fig. 3A). These results indicate that endogenous CREB/CRE signaling is important for Smad-mediated BMP signaling and CRE-mediated enhancement.

Coimmunoprecipitation experiments showed binding of p-Smad1/5/8 to CBP. This result is in accordance with recent reports indicating interaction of CBP/p300 with Smads in both TGF-β and BMP signaling and enhanced transcriptional activity of the Smads complex [15–20].

In the promoter sequence of the *Id1* gene, the CRE and BRE are located in close proximity and are close upstream to the TATA box. BMP and cAMP stimulation might be expected to lead to phosphorylation of Smad1/5/8 by BMPRI and phosphorylation of CREB by PKA, which bind to the BRE and CRE respectively and form the transcriptional cofactor complex regulating transcriptional activity of RNA polymerase II to express the osteoblastic phenotype. As CBP reportedly displays histone acetyl transferase activity and facilitates the transcription of genes [21], the enhancing action of the CRE on the BRE might promote the recruitment of CBP by p-CREB to the transcriptional cofactor complex and CBP might work as a coactivator in Smad-mediated BMP signaling. Further investigations are required to clarify this hypothesis.

Intermittent injection of PTH is known to stimulate bone formation in animals and humans and is currently utilized for the treatment of osteoporosis [22–24]. The mechanism of action for the anabolic effect of daily subcutaneous injection of PTH or its active fragment (teriparatide) on bone has not been elucidated, despite numerous studies. The present results suggest that elevation of intracellular cAMP levels in osteoblastic cells by PTH binding to the PTH1R membrane receptor (a G-protein-coupled receptor that activates the PKA pathway through Gαs stimulation of cAMP production) might enhance endogenous BMP-mediated osteoblastic differentiation by elevated cAMP level. This finding offers a possible explanation for the anabolic effects of the PTH on bone. To date, cAMP-PKA is considered as the main pathway in PTH signaling for the stimulation of osteoblasts, so modulation of BMP signaling by PTH through this pathway represents an important subject of study to further elucidate the mechanisms of action for PTH on bone.

In conclusion, we have demonstrated that the anabolic effects of cAMP on BMP-induced osteoblastic differentiation are mediated through a cAMP-PKA/CREB/CRE signaling pathway. These observations add to the understanding of the osteogenic actions of BMP and form a molecular basis for the development of bone-anabolic drugs that activate the cAMP signaling pathway in osteoblasts, such as PTH.

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References

1. Miyazono K (1999) Signal transduction by bone morphogenetic protein receptors: functional roles of Smad proteins. Bone (NY) 25:91–93
2. Horiuchi H, Saito N, Kinoshita T, Wakabayashi S, Tsutsumimoto T, Takaoka K (2001) Enhancement of bone morphogenetic protein-2-induced new bone formation in mice by the phosphodiesterase inhibitor pentoxifylline. Bone (NY) 28:290–294
3. Tsutsumimoto T, Wakabayashi S, Kinoshita T, Horiuchi H, Takaoka K (2002) A phosphodiesterase inhibitor, pentoxifylline, enhances the bone morphogenetic protein-4 (BMP-4)-dependent differentiation of osteoprogenitor cells. Bone (NY) 31:398–401
4. Horiuchi H, Saito N, Kinoshita T, Wakabayashi S, Tsutsumimoto T, Ohura S, Takaoka K (2004) Enhancement of recombinant human bone morphogenetic protein-2 (rhBMP-2)-induced new bone formation by concurrent treatment with parathyroid hormone and a phosphodiesterase inhibitor, pentoxifylline. J Bone Miner Metab 22:329–334
5. Sasaoka R, Terai H, Toyoda H, Imai Y, Sugiura R, Takaoka K (2004) A prostanoid receptor EP4 agonist enhances ectopic bone formation induced by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 318:804–808
6. Toyoda H, Terai H, Sasaoka R, Oda K, Takaoka K (2005) Augmentation of bone morphogenetic protein-induced bone mass by local delivery of a prostaglandin E EP4 receptor agonist. Bone (NY) 37:555–562
7. Sugiura R, Koike T, Imai Y, Nomura-Furuwatari C, Takaoka K (2006) Bone morphogenetic protein activities are enhanced by 3′,5′-cyclic adenosine monophosphate through suppression of Smad6 expression in osteoprogenitor cells. Bone (NY) 38:206–214
8. Akiyama S, Katagiri T, Namiki M, Yamaji N, Yamamoto N, Miyama K, Shibuya H, Ueno N, Wozney JM, Suda T (1997) Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. Exp Cell Res 235:362–369
9. Katagiri T, Imada M, Yanai T, Suda T, Takahashi N, Kamijo R (2002) Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. Genes Cells 7:949–960
10. Lopez-Rovira T, Chalaux E, Massague J, Rosa JL, Ventura F (2002) Direct binding of Smad1 and Smad4 to two distinct motifs mediates bone morphogenetic protein-specific transcriptional activation of Id1 gene. J Biol Chem 277:3176–3185
11. Korchynskyi O, ten Dijke P (2002) Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem 277:4883–4891
12. Takaoka K, Yoshikawa H, Hashimoto J, Ono K, Matsui M, Nakazato H (1984) Transfilter bone induction by Chinese hamster ovary (CHO) cells transduced by DNA encoding bone morphogenetic protein-4. Clin Orthop Relat Res 300:269–273
13. Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C (1998) A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. Mol Cell Biol 18:967–977
14. Nakagawa K, Imai Y, Ohta Y, Takaoka K (2007) Prostaglandin E, EP4 agonist (ONO-4819) accelerates BMP-induced osteoblastic differentiation. Bone (NY) 41:543–548

15. Nishihara A, Hanai JI, Okamoto N, Yanagisawa J, Kato S, Miyazono K, Kawabata M (1998) Role of p300, a transcriptional coactivator, in signalling of TGF-beta. Genes Cells 3:613–623
16. Feng XH, Zhang Y, Wu RY, Derynck R (1998) The tumor suppressor Smad4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad3 in TGF-beta-induced transcriptional activation. Genes Dev 12:2153–2163
17. Janknecht R, Wells NJ, Hunter T (1998) TGF-beta-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev 12:2114–2119
18. Pouponnot C, Jayaraman L, Massague J (1998) Physical and functional interaction of SMADs and p300/CBP. J Biol Chem 273:22865–22868
19. Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H (2005) Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. J Biol Chem 280:8343–8350
20. Ghosh-Choudhury N, Singha PK, Woodruff K, Stelzer P, Buesel S, Werner SL, Ghosh-Choudhury G (2006) Concerted action of Smad and CREB-binding protein regulates bone morphogenetic protein-2-stimulated osteoblastic colony-stimulating factor-1 expression. J Biol Chem 281:20160–20170
21. Yuan LW, Gamble JE (2001) Histone acetylation by p300 is involved in CREB-mediated transcription on chromatin. Biochim Biophys Acta 1541:161–169
22. Dobnig H, Turner RT (1995) Evidence that intermittent treatment with parathyroid hormone increases bone formation in adult rats by activation of bone lining cells. Endocrinology 136:3632–3638
23. Neer RM, Arnaud CD, Zanchetta JR, Prince R, Gaich GA, Reginster JY, Hodsman AB, Eriksen EF, Ish-Shalom S, Genant HK, Wang O, Mitlak BH (2001) Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N Engl J Med 344:1434–1441
24. Ishizuya T, Yokose S, Hori M, Noda T, Suda T, Yoshiki S, Yamaguchi A (1997) Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells. J Clin Invest 99:2961–2970

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CONSTITUTIVELY ACTIVATED ALK2 AND INCREASED SMAD1/5 COOPERATIVELY INDUCE BMP SIGNALING IN FIBRODYSPLASIA OSSIFICANS PROGRESSIVA

Running title: Heterotopic bone formation in FOP in response to muscle injury

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Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder characterized by congenital malformation of the great toes and by progressive heterotopic bone formation in muscle tissue. Recently, a mutation involving a single amino acid substitution in a bone morphogenetic protein (BMP) type I receptor, ALK2, was identified in patients with FOP. We report here that the identical mutation, R206H, was observed in 19 Japanese patients with sporadic FOP. This mutant receptor, ALK2(R206H) activates BMP signaling without ligand binding. Moreover, expression of Smad1 and Smad5 were up-regulated in response to muscular injury. ALK2(R206H) with Smad1 or Smad5 induced osteoblastic differentiation that could be inhibited by Smad7 or dorsomorphin. Taken together, these findings suggest that the heterotopic bone formation in FOP may be induced by a constitutively activated BMP receptor signaling through Smad1 or Smad5. Gene transfer of Smad7, or inhibition of type I receptors with dorsomorphin may represent strategies for blocking the activity induced by ALK2(R206H) in FOP.

Introduction

Fibrodysplasia ossificans progressiva (FOP; OMIM135100) is a rare autosomal dominant genetic disorder with ectopic bone formation in skeletal muscle tissue (1-4). At birth, most patients with FOP have malformations of the great toes, with hallux valgus, but do not have significant ectopic ossification. Heterotopic bone formation in the muscles begins and other soft tissues begin in early childhood, and is further exacerbated by trauma, surgical treatment, lesional biopsies, and intramuscular injection (4,5).

Ectopic bone formation similar to that observed in FOP is induced by implantation of bone morphogenetic proteins (BMPs) into muscle tissue (6-8). BMPs are members of the transforming growth factor-β (TGF-β) superfamily that were originally isolated from demineralized bone matrix and identified as factors responsible for induction of bone formation (6,7). BMP signaling is transduced by two different types of serine/threonine kinase receptors, termed type I and type II receptors (9,10). The ligand-bound type II receptor activates type I receptor kinase through phosphorylation of the glycine-serine (GS) domain, which is highly conserved among type I BMP and TGF-β receptors. ACVR1/ALK2, BMPR-IA/ALK3, BMPR-IB/ALK6, and ALK1 function as BMP type I receptors. Activated BMP type I receptor kinase activity in turn phosphorylates receptor regulated Smads (R-Smads) including Smad1, Smad5, and Smad8. Phosphorylated R-Smads form heteromeric complexes with Smad4 and translocate into the nucleus to regulate transcription of various target genes, including Id1, which encodes an inhibitor of myogenesis (10-13). Inhibitory Smads (I-Smads), Smad6 and Smad7, are also induced by BMPs. I-Smads inhibit the BMP signaling pathways and thus form a negative feedback loop that down-regulates BMP signaling (14,15). Altered BMP signaling in FOP cells suggests that molecules involved in BMP signaling are responsible for FOP (16-20).

Recently, a recurrent heterozygous mutation in the *ACVR1/ALK2* gene was identified at 617G>A in both familial and sporadic patients with FOP (21,22). This mutation causes an amino acid substitution of Arg to His at codon 206 (R206H) within the GS domain of the ALK2 receptor (21). Although a conformational change in the GS domain leading to activation of the receptor has been suggested to occur, the functional changes of the mutant receptor are still unclear.

In the present study, we report that the common ALK2(R206H) mutation was identified in 19 of 19 Japanese patients with sporadic FOP and determined that ALK2(R206H) constitutively activates BMP signaling in in vitro assays. Expression of ALK2(R206H) in C2C12 myoblasts induced osteoblastic differentiation that was mediated through Smad1 and Smad5, and BMP signaling through ALK2(R206H) could be suppressed by Smad7 or dorsomorphin, two BMP type I receptor inhibitors. We further determined that mRNA levels of Smad1 and Smad5, but not Smad8 or ACVR1/ALK2, are increased in response to muscle injury in vivo. Since heterotopic bone formation in FOP commonly occurs following soft tissue injury, these data support the notion that Smad1 and Smad5 increase following injury further enhances BMP signaling that has been pre-stimulated by a constitutively active ALK2 receptor mutation and leads to heterotopic bone formation. Smad7 and dorsomorphin may represent therapeutic approaches for inhibition of the BMP signaling induced by ALK2(R206H) in FOP.

Materials and Methods

Genomic Sequence

Peripheral blood samples were obtained following informed consent from patients and their relatives in accordance with a protocol approved by the Ethics Committee of Saitama Medical University. Genomic DNA was extracted using a QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany) and exon 4 in the ALK2 gene amplified by polymerase chain reaction (PCR) was directly sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The following oligonucleotides were used as primers:

5'-CCAGTCCTTCTTCCTTCTTCC-3' and

5'-AGCAGATTTTCCAAGTTCCATC-3'.

Cell culture, transfection, and reporter assay

Mouse C2C12 myoblasts and C3H10T1/2 fibroblasts were maintained as described (23,24). HEK293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions (12). Stable ALK2(R206H)-expressing C2C12 cell lines were established by transfecting an expression vector, pcDEF3-ALK2(R206H), and selecting by G418 at 700 mg/ml. BMP-signaling was monitored using IdWT4F-luc or Id985-EGFPd2 reporter plasmids, which express a luciferase and a destabilized enhanced green fluorescent protein, respectively, under the control of a BMP-responsive element in the human *Id1* gene as described previously (12).

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured as a marker of osteoblastic differentiation as described (23,25). In brief, cells were incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$ and 10 mg/ml of *p*-nitrophenylphosphate). After appropriate incubation, reactions were terminated by adding 3 M NaOH and absorbance was measured at 405 nm.

Immunoprecipitation and Immunoblotting

Cells and tissues were lysed in TNE buffer [10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 1% Nonidet P-40]. Immunoblotting was performed using anti-FLAG antibody (clone M2, Sigma, St. Louis, MO), anti-phosphorylated Smad1/5/8 antibody (Cell Signaling, Beverly, MA), anti-V5 antibody (Invitrogen), anti-GFP antibody (GF090R, Nakarai Tesque, Kyoto, Japan), anti-Smad1 antibody (sc-6201, SantaCruz, Santa Cruz, CA), and anti-Smad5 antibody (sc-7443, SantaCruz). Myogenic cells were detected using anti-myosin heavy chain (MHC) antibody (clone MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA) (24).

Induction of muscular injury *in vivo*

To induce muscular injury, 50 µl of habu (*Trimeresurus flavoviridis*) snake venom at 100 µg/kg or vehicle was injected into femoral muscles of 3-week-old C57BL/6 mice. After 3 and 7 days, RT-PCR, real-time quantitative RT-PCR, immunoblotting, and immunohistochemistry were performed. The primers used were as described in Supplemental Table S1. Real-time RT-PCR for Smad1 and Smad5 was performed on Mx3000p (Stratagene, Tokyo, Japan) using QuantiTect Primer Assay (QIAGEN). Muscle tissues were fixed with formalin and embedded in paraffin for immunohistochemical analysis.

Statistical Analysis

Comparisons were made by using student's *t* test. Data were expressed mean ± SD.

Results

Identification of a 617G>A mutation in ALK2 in nineteen Japanese patients with FOP

In order to determine whether FOP in Japanese patients is caused by the same recurrent mutation in ALK2 that was recently reported in familial and sporadic patients with FOP (21), we examined the genomic DNA of 19 Japanese FOP patients. Through DNA sequencing, we confirmed the identical 617G>A (R206H) mutation in the *ACVR1/ALK2* gene in all of the 19 Japanese patients with FOP. No first degree relatives of these patients carried the mutation, indicating that each of the 19 patients are sporadic cases (Supplemental Figure S1).

ALK2(R206H) is a constitutively activated BMP receptor

To examine functional changes of the mutant ALK2 identified in FOP, we examined its intracellular signaling *in vitro*. Transfection of Smad1, a signaling protein specific for the BMP pathway, with ALK2(R206H), but not wild-type ALK2, induced phosphorylation of Smad1 in the absence of BMPs (Fig. 1A). Immuno-detection assays showed that endogenous Smad1/5/8 were phosphorylated and accumulated in nuclei in response to ALK2(R206H) as well as BMPR-IA(Q233D), a form of this BMP type I receptor previously shown to be constitutively active (Fig. 1B) (26). Promoter activity of the *Id1* gene, one of the transcriptional targets of the BMP-Smad axis, was induced by ALK2(R206H) and by BMPR-IA(Q233D) but not wild-type ALK2 in a luciferase assay (Fig. 1C). Induction of the *Id1* promoter by ALK2(R206H) was further confirmed using another construct, Id-EGFPd2 (12) (Fig. 1D and 1E). We additionally examined the effects of ALK2(R206H) on myogenic differentiation, and found that ALK2(R206H) as well as BMPR-IA(Q233D) markedly suppressed myogenesis in C3H10T1/2 cells transfected with a MyoD expression construct (Fig. 1F). Similar suppression of myogenesis by ALK2(R206H) was also observed in C2C12 myoblasts (data not shown). These findings indicate that ALK2(R206H) constitutively activates an intracellular signaling pathway specific for BMPs.

Expression of Smad1 and Smad5 are up-regulated during muscular regeneration

Since muscle tissue injury induces heterotopic bone formation in FOP patients, we hypothesized that receptors or transcription factors that cooperate with ALK2(R206H) in stimulating bone formation are induced in response to muscular injury. To test this hypothesis, we quantified mRNA levels of BMP type I and type II receptors, and of Smads as downstream BMP signaling molecules, during muscular regeneration induced by an intramuscular injection of habu venom in mice. No BMP receptor mRNA levels were changed during muscular regeneration (Fig. 2A). Levels of Smad8, a BMP receptor-regulated Smad, and Smad4, a Co-Smad common to BMP and TGF-β signaling, were not changed during muscular regeneration (Fig. 2B). In contrast, expression levels of two BMP pathway specific Smads, Smad1 and Smad5, were up-regulated as detected by RT-PCR (Fig. 2B), quantitative RT-PCR (Fig. 2C), and immunoblot analysis (Fig. 2D). Levels of Smad1 and Smad5 mRNAs were increased up to ~ 6 ~ 7-fold by days 3 (Fig. 2B and 2C). Smad1 and Smad5 proteins were mainly detected in cells within the regenerating muscle tissues rather than myofibers (Fig. 2E).

To examine the functional interaction of ALK2 and BMP specific Smads, we co-transfected Smad1, Smad5, or Smad8 expression constructs with wild-type ALK2 or ALK2(R206H) into C2C12 myoblasts. Co-transfection and over-expression of Smad1 or Smad5 with ALK2(R206H) increased ALP activity, although enzyme activities were less than those induced by constitutively active BMPR-IA(Q233D) with Smad1 or Smad5 (Fig. 3A). In contrast, co-transfection of Smad8 with ALK2(R206H) induced lower levels of ALP activity than with co-transfection of Smad1 or Smad5, although levels of phosphorylation were not distinguishable among Smad1, Smad5, and Smad8 (Fig. 3A and 3B). Moreover, co-transfection of Smad1 with ALK2(R206H) induced mRNAs related to osteoblastic differentiation such as ALP and osterix, although the levels of mRNAs were lower than that of BMPR-IA(Q233D) (Fig. 3C). However, these mRNAs were not induced by co-transfection of ALK2(R206H) or BMPR-IA(Q233D) with an inactive Smad1 mutant, Smad1(AVA), in which the carboxyl-terminal serine residues phosphorylated by BMP receptors were substituted with alanine residues. These findings suggest that the stimulatory effects of ALK2(R206H) and BMPR-IA(Q233D) on osteoblastic differentiation are mediated through phosphorylation of BMP-specific Smads. The ALP activity induced by ALK2(R206H) and Smad1 was further increased by treatment with BMP-2, BMP-4, BMP-6, or BMP-7 (Fig. 4). Co-transfection of ALK2(R206H) and Smad1 with one of the BMP type II receptors (BMPR-II, ActR-II, or ActR-IIB) further increased ALP acitiviy in the presence and absence of BMPs (data not shown).

Smad7 and dorsomorphin inhibit ALK2(R206H) activity

Addition of a BMP antagonist, noggin that binds to BMPs and blocks their binding to specific receptors in the extracellular space, failed to suppress the ALP activity induced by ALK2(R206H) or BMPR-IA(Q233D) (Fig. 5A). We compared effects of I-Smads on the intracellular signaling induced by ALK2(R206H) and BMPR-IA(Q233D). Both Smad6 and Smad7 at low amounts markedly inhibited the ALP activity induced by BMPR-IA(Q233D), however only Smad7 markedly inhibited signaling by ALK2(R206H), confirming a recent report by Goto et al. (27) (Fig. 5B and 5C).

Recently, the small molecule dorsomorphin was identified as a specific inhibitor of Smad-dependent signaling induced by BMP type I receptors ALK2, BMPR-IA and BMPR-IB (28). Dorsomorphin almost completely inhibited the phosphorylation of FLAG-Smad1 induced by ALK2(R206H) (Fig. 6A). Moreover, dorsomorphin dose-dependently suppressed the ALP activity induced by ALK2(R206H) in C2C12 cells in conditions of both transient and stable over-expression (Fig. 6B and Supplemental Figure S2). ALK2(R206H) was less sensitive to dorsomorphin than BMPR-IA(Q233D) in suppression of ALP activity (Fig. 6B). We established subclonal cell lines of C2C12 myoblasts which stably expressed wild-type ALK2 or ALK2(R206H). Myogenesis of ALK2(R206H)-expressing C2C12 cells was suppressed in the absence of dorsomorphin (Fig. 6C). However, dorsomorphin dose-dependently increased the numbers of MHC-positive myotubes in ALK2(R206H)-expressing C2C12 cells (Fig. 6C).

Discussion

Recently, a recurrent mutation of 617G>A in the *ACVR1/ALK2* gene was identified as the mutation responsible for FOP (21), a rare skeletal disorder associated with heterotopic bone formation in muscle and other soft connective tissues (1-5). In the present study, we identified the same mutation in 19 of 19 Japanese FOP patients. These findings strongly support a causal role of the 617G>A mutation in the pathogenesis of disease for FOP patients with classic FOP.

ALK2 is one of the type I receptors for BMPs, the most potent bone-inducing factors in vertebrates (6,7). The common mutation identified in FOP patients causes a single amino acid