Transgenic Mice overexpressing des-acyl ghrelin show small phenotype. ***Endocrinology.*** **2005**, 146:355-64.

2. 学会発表

国際学会

7 Kangawa K: Ghrelin: discovery and physiological significance. 196th Meeting of the Society for Endocrinology (Plenary lecture), 2005, London.

8 Kangawa K: Ghrelin: Structure and Physiological Significance. The 10th Adiposcience Symposium, 2005, Osaka.

9 Kangawa K: Ghrelin: discovery and physiological significance. The 65th Scientific Sessions American Diabetes Association, 2005, San Diego.

10 Kangawa K: Discovery of ghrelin: physiological significance and translational research. The 2nd International Symposium of the Tohoku University 21st Century COE Program, 2005, Sendai.

国内学会

1. 寒川賢治: 新たな循環調節ペプチドによる展開医療. 第 78 回日本内分泌学会学術総会 (教育講演), 2005, 東京
2. 寒川賢治: 心血管作動性ペプチドの発見とその生理的意義. 第 35 回日本心脈管作動物質学会学術総会 (特別講演), 2005, 宇都宮.
3. 寒川賢治: 新しい生理活性ペプチドの発見とその臨床応用. 日本薬剤学会創立 20 周年記念シンポジウム, 2005, 横浜
4. Kishimoto I, Kangawa K: Molecular mechanism of cardiac protection via natriuretic peptide system. 第 70 回日本循環器学会学術総会, 2005, 名古屋
5. Soeki T, Fukuda Y, Koshiba K, Yamaguchi K, Kawano T, Yamada H, Wakatsuki T, Nomura M, Kangawa K, Itoh S: Ghrelin protects myocardium against ischemia/reperfusion injury via inhibiting myocardial apoptosis. 第 70 回日本循環器学会学術総会, 2005, 名古屋
6. Yoshihara F, Ernst A, Bergmann A, Horio T, Nakamura S, Kishimoto I, Kangawa K, Kawano Y: Midregional proadrenomedullin (MR-ProAM) reflects cardiac dysfunction and predicts mortality in hemodialysis patients with cardiovascular disease. 第 70 回日本循環器学会学術総会, 2005, 名古屋
7. Tokudome T, Kishimoto I, Horio T, Suzuki C, Kohno M, Nakao K, Kawano Y, Kangawa K: Antihypertrophic effect of atrial natriuretic peptide is mediated by activation of regulator of G-protein signaling-4 in cardiac myocytes. 第 70 回日本循環器学会学術総会, 2005, 名古屋

H. 知的財産権の出願・登録状況

国内特許 4 件

国際特許 3 件

厚生労働科学研究費補助金（ヒトゲノム・再生医療等研究事業）
平成17年度分担研究報告書

軟骨細胞におけるC型ナトリウム利尿ペプチド賦活化による作用機構に関する研究

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ANP ファミリーは、これまで水電解質代謝調節など循環器系における重要性が証明されており、特にANP・BNP/GC-A システムについては、ANP、BNP がそれぞれ心房および心室から分泌される循環ホルモンとして心不全や心肥大の最も鋭敏な生化学的マーカーとなること、さらに ANP および BNP が急性心不全の治療薬として有効であることが報告されてきた。以前より ANP ファミリーのうち CNP は骨軟骨由来の細胞にも存在し、作用することが知られていたが、serum amyloid P-component promoter を用いて肝臓で BNP を過剰発現し、血中 BNP 濃度が上昇するトランスジェニックマウスが予想しなかった著明な骨伸長を呈したことより、はじめてＡＮＰファミリーの全く新しい作用としての骨伸長促進作用が明らかとなった。その後、器官培養系を用いて直接ＡＮＰファミリーの骨伸長に対する作用を検討した結果、ＡＮＰファミリーのうち CNP が最も強い骨伸長促進作用をもち、実際、骨軟骨組織に CNP のみが発現していることが確認された。続いて作成された CNP 遺伝子欠損マウスでは骨の伸長障害が、軟骨特異的に CNP を過剰発現するトランスジェニックマウスでは骨の伸長促進が認められ、ANP、BNP、GC-A の各遺伝子欠損マウスに骨格異常が認められないこととあわせて骨の伸長促進には CNP-GC-B 系が重要であることが明らかとなった。CNP 遺伝子欠損マウスでは内軟骨性骨化の障害による骨の伸長障害が確認され、CNP が生理的にも内軟骨性骨化による骨伸長に不可欠な因子であることが証明された。CNP 遺伝子欠損マウスにおいて、成長板の増殖細胞層および肥大化細胞層の狭小が認められる。CNP 遺伝子欠損マウスの成長板において軟骨細胞分化マーカーである II 型コラーゲン、X 型コラーゲン、インディアンヘッジホッグ（Ihh）の発現領域は成長板各層の増減に応じて変化するものの、その発現パターンや発現量には大きな変化がみられないことから、CNP は PTHrP や Ihh のように成長板軟骨細胞の分化には影響を与えないことが考えられている。実際、CNP 遺伝子欠損マウスでは成長板軟骨の著明な短縮をきたすが、軟骨細胞の分化に異常を認めず、二次骨化中心の出現も正常であった。

A. 研究目的

ナトリウム利尿ペプチドファミリーは３種類のペプチドホルモン、心房性ナトリウム利尿ペプチド(ANP)、脳性ナトリウム利尿ペプチド(BNP)、C 型ナトリウム利尿ペプチド(CNP)によって構成される。ANP, BNP はそれぞれ主に心房、心室から生合成、分泌され、共通の受容体であるグアニル酸シクラーゼ(guanylyl cyclase, GC-A)を介して、利尿作用、ナトリウム利尿作用、血管平滑筋弛緩作用に基づく血圧降下作用、さらにはアルドステロン分泌抑制作用など、多彩な生物作用を発現する。一方、CNP は全身に広く分布し、ANP, BNP とは異なる受容体である GC-B を介し、局所調節因子として作用すると考えられてきたが、その本質は不明であった。われわれはこれまでに肝臓でナトリウム利尿ペプチドを過剰発現させることにより、血中 BNP 濃度を約 100 倍に上昇させた BNP 過剰発現トランスジェニックマウスを作製し、BNP 過剰発現トランスジェニックマウスにおいて野生型に比較して収縮期血圧の低下とともに、脊椎の湾曲および尻尾、四肢の伸長が認められた。このことから、ナトリウム利尿ペプチドの内軟骨性骨化における意義に注目し、CNP の生理的、病態的意義を個体レベルで検討する目的で CNP ノックアウトマウスを作製した。

B. 研究方法

軟骨無形成症は四肢短縮型小人症の代表的疾患で頻度は15,000～40,000出生に１人とされ、骨形成異常疾患の中では最も頻度が高い。治療として

軟骨無形成症患者の著しい低身長に対し、脚延長術が行われることが多いが、現在では成長ホルモン治療も行われている。染色体4q13に位置する3型線維芽細胞増殖因子受容体fibroblast growth factor receptor 3 (FGFR3)の遺伝子の異常が原因であることが明らかになり、その変異部位は非常に均質で膜貫通領域の380番目のグリシンのアルギニンへの置換変異(G380R)が大部分の症例で観察された。この変異は日本人においてもhot spotとして認められ、この変異受容体はFGFリガンド非存在下で受容体の活性化がおこること、また同時にFGFリガンド刺激により、さらなる活性化がおこることが証明されている。われわれはマウスCNP遺伝子翻訳領域全長を含むexon 1とexon 2をネオマイシン耐性遺伝子で置換したターゲティングベクターを作成し、この導入遺伝子との相同組み替えによりノックアウトアリルが確認されたES細胞を、C57BL/6マウス胚へ移植し、キメラマウスを得た。このキメラマウスの交配により、ヘテロ及びホモのノックアウトマウスが得られた。小脳及び軟骨におけるCNP発現をRNA プロテクションアッセイにて検討したところ、野生型で認められるCNP遺伝子発現がホモ接合型であるNppc$^{-/-}$マウスでは消失していた。これまでの解析からNppc$^{-/-}$マウスは四肢、体幹の著明な短縮を認め、組織学的にも成長板軟骨の短縮を認め、内軟骨性骨化により形成される長管骨の短縮を認めることから、四肢短縮型小人症のモデル動物となることを明らかにした。CNPの治療薬としての効果を検討する目的で、循環血液中のCNPを種々の濃度に上昇させるCNP過剰発現マウス(SAP-CNP-Tg)を作製し、CNPの全身投与と同様の状況を遺伝子改変動物で再現する。このSAP-CNP-Tgマウスを今回作成してCNP-KOマウス(*Nppc*$^{-/-}$)と交配させて、SAP-CNP-Tg /*Nppc*$^{-/-}$マウスを作製した。SAP-CNP-Tg およびSAP-CNP-Tg /*Nppc*$^{-/-}$マウスの表現型を成長曲線、全身の軟レントゲン撮影にて解析する。SAP-CNP-Tgを用いてCNPの成長板軟骨への作用、関節軟骨への作用を組織学的、in situ hybridization による種々の軟骨細胞の分化マーカーの解析により検討する。また、SAP-CNP-Tg を用いてCNPを全身投与する場合の用量設定、有効性、軟骨以外の臓器への作用等全身への影響、安全性を検討する。

(倫理面への配慮)

動物実験に関しては京都大学動物実験委員会に申請し、承認を得ている(MedKyo01094)。組み替えDNA実験計画について京都大学の承認を得ている。ヒトの血液、関節液検体、CNP 関連遺伝子解析に関しては患者、その他関係者の人権及び利益の保護の取扱について十分配慮した研究計画書を作製し、京都大学大学院医学研究科・医の倫理委員会の承認を得ている(G20-3)。

C. 研究結果

雌性 10 週齢のホモ接合型の CNP$^{-/-}$マウスの外観は明らかな体長の短躯、四肢の短縮を認めた。一方、ヘテロ接合型は明らかな外観の変化を認めなかった。成長曲線においてもヘテロ接合型においては野生型と差を認めないが、ホモ接合型のNppc$^{-/-}$マウスにおいては生後1週齢において体長

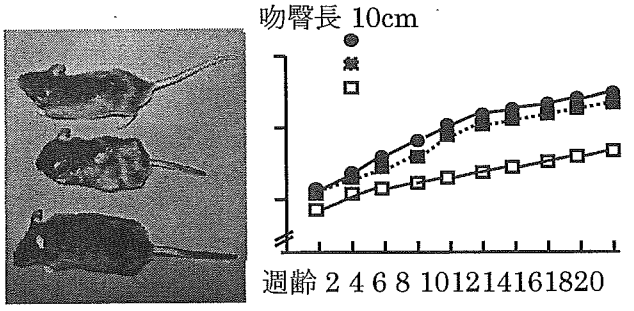

図1 左：上、野生型、中、CNP ノックアウトマウス(Nppc-/-)、下、CNP ノックアウトトランスジェニックマウス(SAP-CNP-Tg /Nppc-/-)の外観、右：3 系統マウスの成長曲線；●野生型、■SAP-CNP-Tg /Nppc-/-、□Nppc-/-

の有意差を認め、その差は 10 週齢まで成長とともに拡大を認めた（図 1)。軟 X 線写真においても Nppc$^{-/-}$マウスでは椎体および四肢長管骨の短縮が認められた（図 2)。骨計測値でも内軟骨性骨化において長さが規定される脛骨、大腿骨、腰椎の長軸長および頭蓋骨前後径が Nppc$^{-/-}$マウスにおいて野生型に比較し、約 50～80%に短縮していた。一方、膜性骨化により長さが規定される頭蓋骨横径には有意差は認められず、Nppc$^{-/-}$マウスでは内軟骨性骨化が障害されていることが示唆された（図 2)。

内軟骨性骨化の過程では軟骨細胞は骨端部から骨幹部に向かい、静止軟骨細胞層、増殖軟骨細胞

層、前肥大化軟骨細胞層、肥大化軟骨細胞層へと分化していくが、生後7日齢の脛骨成長板軟骨

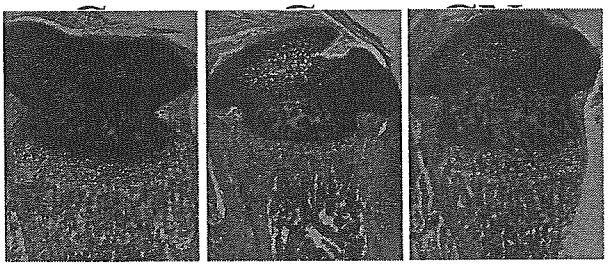

図2　CNP ノックアウトマウスの成長板軟骨組織像。左、雌性7週齢の野生型マウス、中、ホモ接合型(Nppc-/-)、および右、SAP-CNP-Tg /Nppc-/-マウスの脛骨成長板軟骨組織の alcian blue- H&E 染色像。拡大率 x40。

組織像において、Nppc$^{-/-}$マウスでは増殖軟骨細胞層、前肥大化軟骨細胞層、および肥大化軟骨細胞層の短縮が認められた（図 3a, b)。さらに、2型コラーゲン、X型コラーゲン等の軟骨分化マーカーの発現についても in situ hybridization 法により検討したが、いずれも Nppc$^{-/-}$マウスでは発現部位の縮小が認められた。われわれはさらに CNP 投与モデルとして血中 CNP 濃度の上昇したトランスジェニックマウス(SAP-CNP-Tg)を作製し CNP 濃度依存性の四肢、体幹の過剰伸長を観察した。組織学的には成長板軟骨及び関節軟骨の肥大を観察し、この発現型が軟骨に CNP を過剰発現するトランスジェニックマウス(col2-CNP-Tg)と同様であることから、血中 CNP が成長板軟骨及び関節軟骨に作用し、肥大化を引き起こすことを明らかにした。in situ hybridization の検討から CNP、GC-B の発現と2型コラーゲンの発現部位はほぼ一致していたこと、CNP 投与により cyclic GMP が上昇し、関節軟骨を肥大化することから、関節軟骨に CNP/GC-B 系が発現し、CNP が関節軟骨においても有効であることを明らかにした。成長板軟骨、関節軟骨において組織学的解析、軟骨分化マーカーとして 2 型コラーゲン、10 型コラーゲン、Runx2, Indian hedgehog の解析を行い、野生型と比較し、SAP-CNP-Tg マウスの肥大した成長板軟骨、関節軟骨が正常の構造を保っていること明らかにした。

CNP ノックアウトマウスにおいて SAP プロモーターにて軟骨特異的に CNP 過剰発現させたところ、椎体および四肢長管骨の短縮、成長板における増殖軟骨細胞層、前肥大化軟骨細胞層、および肥大化軟骨細胞層の短縮が完全に是正され、正常マウスと同等の表現型を示すことを証明した（図2)。また、CNP が変異 FGFR3(G380R)を持つ軟骨異栄養症のモデルマウスにおいても同様の効果を持つことを明らかにしており、CNP/GC-B 系の賦活化が軟骨異栄養症に対する新しいカテゴリーの治療法となる可能性が示されてきている。

D. 考察

今回の実験結果から CNP による軟骨肥大化作用は FGFR3 刺激による軟骨成長抑制に対して拮抗することが示された。FGFR3 の活性型変異が内軟骨性骨化を障害し、軟骨無形成症の原因であり、疾患モデルマウスを用いた検討において CNP が軟骨無形成症の四肢体幹の短縮を改善したことの、メカニズムが明らかとなった。また、常染色体劣性遺伝による四肢短縮型の低身長をきたす疾患として知られていた Maroteaux 型遠位中間肢異形成症 において GC-B の不活性型遺伝子変異が同定され、初めて CNP/GC-B 系がヒトの骨系統疾患の原因であることが証明された。CNP/GC-B 系がヒトの骨・軟骨における重要性が確認されたことから、CNP/GC-B 系は骨・軟骨における新しい制御機構として注目されている。

E. 結論

本研究により、成長板軟骨と関節軟骨における CNP/GC-B 系の機能的意義とその分子機構を解明し、軟骨再生を標的とする CNP/GC-B 系のトランスレーショナルリサーチが推進され、軟骨無形成症を含む先天性骨軟骨疾患のみならず、変形性関節症等の関節軟骨病変に対する新規治療法の開発につながることが期待される。

F. 健康危険情報

なし

G. 発表

1. 論文発表

1. Majima T, Komatsu Y, Doi K, Takagi C, Shigemoto M, Morimoto T, Corners J, Nakao K, Negative correlation between bone mineral density and TSH receptor antibodies in male patients with untreated Graves' disease, ***Osteoporos Int,*** *in press.*

2. Nishida N, Komatsu Y, Komeda T, Fukuda Y, Interferon-α improves bone resorption and osteopenia in patients with chronic hepatitis C. ***Hepatology Res.2006***, 34:222-227.
3. Majima T, Komatsu Y, Doi K, Takagi C, Shigemoto M, Morimoto T, Corners J, Nakao K, Clinical significance of risedronate for osteoporosis in the initial treatment of male patients with Graves' disease. ***J Bone Miner Metab 2006***, 24:105-113.
4. Nambu T, Arai H, Komatsu Y, Yasoda A, Moriyama K, Kanamoto N, Itoh H, Nakao K, Expression of the aderenomedullin gene in adipose tissue, ***Regul Pept 2005***, 132:17-22.
5. Majima T, Komatsu Y, Doi K, Shigemoto M, Takagi C, Fukao A, Kojima M, Tamaki H, Ito J, Nakao K, Anaplastic thyroid carcinoma associated with Graves' disease, ***Endocrinol J 2005***, 52:551-557.
6. Majima T, Doi K, Komatsu Y, Itoh H, Fukao A, Shigemoto M, Takagi C, Corners J, Mizuta N, Kato R, Nakao K, Papillary thyroid carcinoma without metastases manifesting as an autonomously functioning thyroid nodule, ***Endocrin J 2005***, 52:309-316.
7. Ozasa A, Komatsu Y, Yasoda A, Miura M, Nakatsuru Y, Sakuma Y, Arai H, Itoh N, Nakao N, Complementary antagonistic actions between C-Type natriuretic peptide and MAPK pathway through FGFR-3 in ATDC5 Cells. ***Bone 2005***, 36:1056-1064.
8. Furuto-Kato S, Matsukura S, Ogata M, Azuma N, Manabe T, Shigeno C, Asato R, Tanaka K, Komatsu Y, Nakao K, Primary hyperparathyroidism presumably caused by chronic parathyroiditis manifesting from hypocalcemia to severe hypercalcemia. ***Intern Med. 2005***, 44:60-64.
9. Majima T, Komatsu Y, Yamada T, Koike Y, Shigemoto M, Takagi C, Hatanaka I, Nakao K, Decreased bone mineral density at the distal radius, but not at the lumbar spine or the femoral neck, in Japanese type 2 diabetic patients. ***Osteoporos Int 2005***, 16:907-913.

2. 学会発表

国際学会

1. Komatsu Y, Yasoda A, Tamura N, Nakatsuru Y, Arai H, Nakao K, CNP/guanylyl cyclase B (GC-B) system regulates endochondral ossification – the analysis of GC-B null mice, 27th American Society for Bone and Mineral Research, Sept23-27, 2005, Nashuville, Tennessee, USA.
2. Majima T, Komatsu Y, Doi K, Shigemoto M, Takagi C, Nakao K, Reduced bone mineral density in male patients with untreated graves' Disease, 27th American Society for Bone and Mineral Research, Sept23-27, 2005, Nashuville, Tennessee, USA.

国内学会

1. 八十田明宏、小松弥郷、中所英樹、小笹亜美、中津留有子、田村尚久、荒井宏司、小川佳宏、中尾一和、骨系統疾患に対する CNP/GC-B 系の治療的意義、第 102 回日本内科学会総会、2005/4/7-9、大阪
2. 小松弥郷、骨粗鬆症と糖尿病、講演（万有製薬）、2005/4/13、京都
3. 真嶋隆文、高木千恵子、本田久子、重本道香、土居健太郎、小松弥郷、初診時男性バセドウ病患者の骨密度の検討、第 3 回臨床骨代謝懇話会、2005/4/23、京都
4. 真嶋隆文、土居健太郎、高木千恵子、重本道香、深尾篤嗣、小松弥郷、DXA 使用における 2 型糖尿病患者の骨密度の検討、第 48 回日本糖尿病学会、2005/5/12-14、大阪
5. 南部拓央、荒井宏司、八十田明宏、森山賢治、小松弥郷、中尾一和、高脂肪食負荷マウスにおける脂肪組織由来アドレノメデュリン、第 48 回日本糖尿病学会、2005/5/12-14、大阪
6. 小松弥郷、SERM による骨粗鬆症治療の新展開、産婦人科診療内容向上会、2005/5/21、京都
7. 小松弥郷、病診連携としての骨粗鬆症外来の取り組み、KOCP～内科医のための骨粗鬆症カンファレンス～、2005/5/28、京都
8. 真嶋隆文、小松弥郷、高木千恵子、重本道香、土居健太郎、中尾一和、２型糖尿病女性における低用量ピオグリタゾンの有用性の検討、

第 6 回糖尿病と生活習慣病治療研究会、2005/5/28、京都

9. 小松弥郷、拡大する骨粗鬆症治療薬選択の現状と展望、福井県病院薬剤師講演会、2005/6/28、福井
10. 小松弥郷、八十田明宏、佐久間陽子、小笹亜美、中津留有子、荒井宏司、中尾一和、Risedronate投与後の骨粗鬆症患者の骨代謝マーカーと骨密度の変化についての検討-Cyclic Etidronate との比較、第 78 回日本内分泌学会学術総会、2005/7/1-3、東京
11. 八十田明宏、小松弥郷、小笹亜美、中津留有子、田村尚久、荒井宏司、小川佳宏、中尾一和、マウス生体に対する CNP 投与による骨伸長促進作用の検討、第 78 回日本内分泌学会学術総会、2005/7/1-3、東京
12. 中津留有子、小松弥郷、八十田明宏、小笹亜美、佐久間陽子、荒井宏司、中尾一和、成長版軟骨における Na 利尿ペプチド系の意義－BNP トランスジェニックマウスを用いた検討、第 78 回日本内分泌学会学術総会、2005/7/1-3、東京
13. 小松弥郷、高カルシウム血症の臨床アップデート、第 78 回日本内分泌学会学術総会クリニカルアワー、2005/7/1-3、東京
14. 小松弥郷、八十田明宏、佐久間陽子、小笹亜美、中津留有子、荒井宏司、中尾一和、CNP/guanylyl cyclase B(GC-B)系による内軟骨性骨化調節機構の検討- GC-B 遺伝子欠損マウスを用いた解析、第 23 回日本骨代謝学会、2005/7/21-23、大阪
15. 八十田明宏、小松弥郷、中所英樹、小笹亜美、中津留有子、藤井寿人、田村尚久、荒井宏司、小川佳宏、中尾一和、CNP－GC-B 系の賦活化による骨伸長障害治療への展開研究、第 42 回日本臨床分子医学会学術集会、2005/7/22-23、京都
16. 小笹亜美、小松弥郷、八十田明宏、中津留有子、荒井宏司、伊藤信行、中尾一和、培養軟骨細胞における CNP と FGF18 との相互作用についての検討、第 42 回日本臨床分子医学会学術集会、2005/7/22-23、京都
17. 平田雅一、荒井宏司、藤井寿人、中津留有子、南部拓央、金本巨哲、有安宏之、森山賢治、八十田明宏、小松弥郷、田上哲也、臼井健、島津章、中尾一和、甲状腺ホルモン不応症の一例、第 38 回間脳下垂体疾患研究会、2005/8/27、京都
18. 小鳥真司、小松弥郷、森山賢治、八十田明宏、金本巨哲、南部拓央、中津留有子、藤井寿人、荒井宏司、中尾一和、後腹膜線維症を合併したリンパ球性下垂体炎の一例、第 69 回京都内分泌同好会プログラム、2005/9/3、京都
19. 小松弥郷、骨粗鬆症とカルシウム代謝の臨床アップデート、中京東部医師会学術講演会、2005/9/15、京都
20. 小松弥郷、八十田明宏、田村尚久、中津留有子、藤井寿人、荒井宏司、中尾一和、CNP/guanylyl cyclase B(GC-B)系による骨形成作用の検討-GC-B 遺伝子欠損マウスを用いた解析、第 7 回日本骨粗鬆症学会、2005/10/13-15、大阪
21. 真嶋隆文、小松弥郷、高木千恵子、重本道香、土居健太郎、中尾一和、2 型糖尿病患者における骨密度減少と HbA1c との関係についての検討、第 7 回日本骨粗鬆症学会、2005/10/13-15、大阪
22. 八十田明宏、小松弥郷、小笹亜美、中津留有子、藤井寿人、田村尚久、荒井宏司、中尾一和、マウスに対する CNP 投与による骨伸長促進作用の検討、第 55 回関西カルシウム懇話会、2005/10/22、大阪
23. 小松弥郷、平田雅一、八十田明宏、金本巨哲、森山賢治、南部拓央、中津留有子、藤井寿人、荒井宏司、安里亮、松本繁己、中尾一和、塩酸アムルビジンと放射線照射により約 1 年間治療した甲状腺未分化癌の 1 例、第 48 回日本甲状腺学会、2005/11/21-23、東京
24. 真嶋隆文、小松弥郷、高木千恵子、重本道香、土居健太郎、深尾篤嗣、中尾一和、男性バセドウ病患者の初診時骨密度の検討、第 48 回日本甲状腺学会、2005/11/21-23、東京
25. 小松弥郷、カルシウム代謝異常症と骨粗鬆症の臨床 UpDate、KOCP～内科医のための骨粗鬆症カンファレンス～、2005/12/10、京都
26. 中川和彦、吉田健司、八十田明宏、田村尚久、藤井寿人、中津留有子、南部拓央、金本巨哲、森山賢治、小松弥郷、荒井宏司、細田公則、向山政志、伊藤裕、中尾一和、中枢性交感神経抑制薬が著効を奏したレニン性高血圧症

の一例、 第 33 回関西高血圧研究会、2005/1/27、京都

27. 西城さやか、荒井宏司、田部有香、伴健二、藤井寿人、中津留有子、南部拓央、金本巨哲、森山賢治、八十田明宏、小松弥郷、中尾一和、東達也、抗甲状腺薬による薬剤性血小板減少症と特発性血小板減少性紫斑病を合併した Graves' disease の一例、第 28 回京都甲状腺研究会、2005/1/28、京都

28. 髭 秀樹、伴健二、八十田明宏、藤井寿人、中津留有子、南部拓央、金本巨哲、森山賢治、原田昌樹、小松弥郷、荒井宏司、丹原圭一、三上幸雄、中尾一和、下垂体機能低下症と腹部大動脈瘤をきたした Multifocal Systematic Fibrosis の一例、第 70 回京都内分泌同好会、2006/2/25、京都

29. 小松弥郷、パネルディスカッション「ステロイド骨粗鬆症への対応」、第 12 回近畿骨粗鬆症学研究会、2006/2/25、大阪

30. 平田雅一、小松弥郷、金本巨哲、南部拓央、中津留有子、荒井宏司、森徹、安里亮、松本繁己、中尾一和、甲状腺未分化癌に対し塩酸アムルビシンと放射線照射により約 1 年間フォローしえた一例、第 16 回臨床内分泌代謝 Update、2006/2/25-26、石川

31. 藤澤武、小松弥郷、八十田明宏、金本巨哲、南部拓央、中津留有子、藤井寿人、森山賢治、荒井宏司、細田公則、中尾一和、検診にて偶然発見された家族性褐色細胞腫の一例、第 16 回臨床内分泌代謝 Update、2006/2/25-26、石川

32. 小松弥郷、八十田明宏、金本巨哲、南部拓央、中津留有子、藤井寿人、森山賢治、荒井宏司、中尾一和、副腎偶発腫を契機に診断された神経線維腫症 I 型の一例、第 16 回臨床内分泌代謝 Update、2006/2/25-26、石川

33. 小鳥真司、小松弥郷、森山賢治、八十田明宏、金本巨哲、南部拓央、中津留有子、藤井寿人、荒井宏司、中尾一和、リンパ球性下垂体炎、後腹膜線維症、肥大型心筋症を合併した稀な一例、第 16 回臨床内分泌代謝 Update、2006/2/25-26、石川

34. 小鳥真司、小松弥郷、森山賢治、八十田明宏、金本巨哲、南部拓央、中津留有子、藤井寿人、荒井宏司、中尾一和、リンパ球性下垂体炎、後腹膜線維症、肥大型心筋症を合併した稀な一例、16th 臨床内分泌 update、2006/3/25-26、金沢

H. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧

雑誌　（中尾一和、小松弥郷）

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ozasa A, Komatsu Y, Yasoda A, Miura M, Sakuma Y, Nakatsuru Y, Arai H, Itoh N, Nakao K	Complementary antagonistic actions between C-type natriuretic peptide and the MAPK pathway through FGFR-3 in ATDC5 cells.	***Bone***	36	1056-64	2005
Natsui K, Tanaka K, Suda M, Yasoda A, Sakuma Y, Ozasa A, Ozaki S, Nakao K	High-dose glucocorticoid treatment induces rapid loss of trabecular bone mineral density and lean body mass.	***Osteoporos Int***	17	105-108	2006
Majima T, Komatsu Y, Doi K, Shigemoto M, Takagi C, Fukao A, Kojima M, Tamaki H, Ito J, Nakao K	Anaplastic thyroid carcinoma associated with graves' disease.	***Endocr J***	52	551-7	2005
Nambu T, Arai H, Komatsu Y, Yasoda A, Moriyama K, Itoh H, Nakao K	Expression of the adrenomedullin gene in adipose tissue.	***Regul Pept***	132	17-22	2005
Yura S, Itoh H, Sagawa N, Yamamoto H, Masuzaki H, Nakao K, Kawamura M, Takemura M, Kakui K, Ogawa Y, Fujii S	Role of premature leptin surge in obesity resulting from intrauterine undernutrition.	***Cell Metab***	1	371-8	2005
Majima T, Doi K, Komatsu Y, Itoh H, Fukao A, Shigemoto M, Takagi C, Corners J, Mizuta N, Kato R, Nakao K	Papillary thyroid carcinoma without metastases manifesting as an autonomously functioning thyroid nodule.	***Endocr J***	52	309-16	2005
Nakanishi M, Saito Y, Kishimoto I, Harada M, Kuwahara K, Takahashi N, Kawakami R, Nakagawa Y, Tanimoto K, Yasuno S, Usami S, Li Y, Adachi Y, Fukamizu A, Garbers DL, Nakao K	Role of natriuretic peptide receptor guanylyl cyclase-A in myocardial infarction evaluated using genetically engineered mice.	***Hypertension***	46	441-7	2005
Tokudome T, Horio T, Kishimoto I, Soeki T, Mori K, Kawano Y, Kohno M, Garbers DL, Nakao K, Kangawa K	Calcineurin-nuclear factor of activated T cells pathway-dependent cardiac remodeling in mice deficient in guanylyl cyclase A, a receptor for atrial and brain natriuretic peptides.	***Circulation***	111	3095-104	2005
Furuto-Kato S, Matsukura S, Ogata M, Azuma N, Manabe T, Shigeno C, Asato R, Tanaka K, Komatsu Y, Nakao K	Primary hyperparathyroidism presumably caused by chronic parathyroiditis manifesting from hypocalcemia to severe hypercalcemia.	***Intern Med***	44	60-4	2005

Iwakura H, Hosoda K, Son C, Fujikura J, Tomita T, Noguchi M, Ariyasu H, Takaya K, Masuzaki H, Ogawa Y, Hayashi T, Inoue G, Akamizu T, Hosoda H, Kojima M, Itoh H, Toyokuni S, Kangawa K, Nakao K	Analysis of rat insulin II promoter-ghrelin transgenic mice and rat glucagon promoter-ghrelin transgenic mice.	***J Biol Chem***	280	15247-56	2005
Saito T, Itoh H, Yamashita J, Doi K, Chun TH, Tanaka T, Inoue M, Masatsugu K, Fukunaga Y, Sawada N, Sakaguchi S, Arai H, Tojo K, Tajima N, Hosoya T, Nakao K	Angiotensin II suppresses growth arrest specific homeobox (Gax) expression via redox-sensitive mitogen-activated protein kinase (MAPK).	***Regul Pept***	127	159-67	2005
Tanaka T, Fukunaga Y, Itoh H, Doi K, Yamashita J, Chun TH, Inoue M, Masatsugu K, Saito T, Sawada N, Sakaguchi S, Arai H, Nakao K	Therapeutic potential of thiazolidinediones in activation of peroxisome proliferator-activated receptor gamma for monocyte recruitment and endothelial regeneration.	***Eur J Pharmacol***	508	255-65	2005
Takahashi N, Saito Y, Kuwahara K, Harada M, Tanimoto K, Nakagawa Y, Kawakami R, Nakanishi M, Yasuno S, Usami S, Yoshimura A, Nakao K	Hypertrophic responses to cardiotrophin-1 are not mediated by STAT3, but via a MEK5-ERK5 pathway in cultured cardiomyocytes.	***J Mol Cell Cardiol***	38	185-92	2005
Majima T, Komatsu Y, Yamada T, Koike Y, Shigemoto M, Takagi C, Hatanaka I, Nakao K	Decreased bone mineral density at the distal radius, but not at the lumbar spine or the femoral neck, in Japanese type 2 diabetic patients.	***Osteoporos Int***	16	907-13	2005
Majima T, Komatsu Y, Doi K, Shigemoto M, Takagi C, Fukao A, Kojima M, Tamaki H, Ito J, Nakao K	Anaplastic thyroid carcinoma associated with graves' disease.	***Endocr J***	52	551-7	2005
Tanimoto K, Saito Y, Hamanaka I, Kuwahara K, Harada M, Takahashi N, Kawakami R, Nakagawa Y, Nakanishi M, Adachi Y, Shirakami G, Fukuda K, Yoshimura A, Nakao K	SOCS1/JAB likely mediates the protective effect of cardiotrophin-1 against lipopolysaccharide-induced left ventricular dysfunction in vivo.	***Circ J***	69	1412-7	2005
Tanaka T, Fukunaga Y, Itoh H, Doi K, Yamashita J, Chun TH, Inoue M, Masatsugu K, Saito T, Sawada N, Sakaguchi S, Arai H, Nakao K	Therapeutic potential of thiazolidinediones in activation of peroxisome proliferator-activated receptor gamma for monocyte recruitment and endothelial regeneration.	***Eur J Pharmacol***	508	255-65	2005
Majima T, Komatsu Y, Doi K, Takagi C, Shigemoto M, Morimoto T, Corners J, Nakao K	Clinical significance of risedronate for osteoporosis in the initial treatment of male patients with Graves' disease.	***J Bone Miner Metab***	24	105-113	2006
Nishida N, Komatsu Y, Komeda T, Fukuda Y	Interferon- α improves bone resorption and osteopenia in patients with chronic hepatitis C.	***Hepatology Res***	34	222-227	2006

雑誌　(寒川賢治)

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Soeki T, Kishimoto I, Okumura H, Tokudome T, Horio T, Mori K, Kangawa K.	C-type natriuretic peptide, a novel antifibrotic and antihypertrophic agent, prevents cardiac remodeling after myocardial infarction.	***J Am Coll Cardiol***	45	608-16	2005
Fukuhara S, Sakurai A, Sano H, Yamagishi A, Somekawa S, Takakura N, Saito Y, Kangawa K, Mochizuki N.	Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway.	***Mol Cell Biol***	25	136-46	2005
Mori K, Miyazato M, Ida T, Murakami N, Serino R, Ueta Y, Kojima M, Kangawa K.	Identification of neuromedin S and its possible role in the mammalian circadian oscillator system.	***EMBO J***	24	325-35	2005
Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y, Fujii T, Uematsu M, Ohgushi H, Yamagishi M, Tokudome T, Mori H, Miyatake K, Kitamura S.	Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy.	***Circulation***	112	1128-35	2005
Makino T, Matsumoto M, Suzuki Y, Kitajima Y, Yamamoto K, Kuramoto M, Minamitake Y, Kangawa K, Yabuta M.	Semisynthesis of human ghrelin: Condensation of a Boc-protected recombinant peptide with a synthetic O-acylated fragment.	***Biopolymers***	79	238-47	2005
Ida T, Mori K, Miyazato M, Egi Y, Abe S, Nakahara K, Nishihara M, Kangawa K, Murakami N.	Neuromedin s is a novel anorexigenic hormone.	***Endocrinology***	146	4217-23	2005
Date Y, Toshinai K, Koda S, Miyazato M, Shimbara T, Tsuruta T, Niijima A, Kangawa K, Nakazato M	Peripheral interaction of ghrelin with cholecystokinin on feeding regulation.	***Endocrinology***	146	3518-25	2005
Hanabusa K, Nagaya N, Iwase T, Itoh T, Murakami S, Shimizu Y, Taki W, Miyatake K, Kangawa K	Adrenomedullin enhances therapeutic potency of mesenchymal stem cells after experimental stroke in rats.	***Stroke***	36	853-8	2005
Nishi Y, Hiejima H, Mifune H, Sato T, Kangawa K, Kojima M	Developmental changes in the pattern of ghrelin's acyl modification and the levels of acyl-modified ghrelins in murine stomach.	***Endocrinology***	146	2709-15	2005
Yoshihara F, Suga S, Yasui N, Horio T, Tokudome T, Nishikimi T, Kawano Y, Kangawa K	Chronic administration of adrenomedullin attenuates the hypertension and increases renal nitric oxide synthase in Dahl salt-sensitive rats.	***Regul Pept***	128	7-13	2005
Nishi Y, Hiejima H, Hosoda H, Kaiya H, Mori K, Fukue Y, Yanase T, Nawata H, Kangawa K, Kojima M	Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin.	***Endocrinology***	146	2255-64.	2005
Iwase T, Nagaya N, Fujii T, Itoh T, Ishibashi-Ueda H, Yamagishi M, Miyatake K, Matsumoto T, Kitamura S, Kangawa K	Adrenomedullin enhances angiogenic potency of bone marrow transplantation in a rat model of hindlimb ischemia.	***Circulation***	111	356-62	2005

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Complementary antagonistic actions between C-type natriuretic peptide and the MAPK pathway through FGFR-3 in ATDC5 cells

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Abstract

We previously reported that C-type natriuretic peptide (CNP) stimulates endochondral ossification and corrects the reduction in body length of achondroplasia model mouse with constitutive active fibroblast growth factor receptor 3 (FGFR-3). In order to examine the interaction between CNP and FGFR-3, we studied intracellular signaling by using ATDC5 cells, a mouse chondrogenic cell line, and found that FGF2 and FGF18 markedly reduced CNP-dependent intracellular cGMP production, and that these effects were attenuated by MAPK inhibitors. Western blot analysis demonstrated that the level of GC-B, a particulate guanylyl cyclase specific for CNP, was not changed by treatment with FGFs. Conversely, CNP and 8-bromo-cGMP strongly and dose-dependently inhibited the induction of ERK phosphorylation by FGF2 and FGF18 without changing the level of FGFR-3, although they did not affect the phosphorylation of STAT-1. In the organ-cultured fetal mouse tibias, CNP and FGF18 counteracted on the longitudinal bone growth, and both the size and number of hypertrophic chondrocytes. The FGF/FGFR-3 pathway is known as the negative regulator of endochondral ossification. We found that FGFs inhibited CNP-stimulated cGMP production by disrupting the signaling pathway through GC-B while CNP antagonized the activation of the MAPK cascade by FGFs. These results suggest that the CNP/GC-B pathway plays an important role in growth plate chondrocytes and constitutes the negative cross talk between FGFs and the activity of MAPK. Our results may explain one of the molecular mechanisms of the growth stimulating action of CNP and suggest that activation of the CNP/GC-B pathway may be effective as a novel therapeutic strategy for achondroplasia.

Keywords: Natriuretic peptide; Guanylyl cyclase; Chondrocyte; FGF; MAPK

Introduction

The natriuretic peptide family consists of three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) [1]. They can influence a variety of homeostatic processes by accumulation of the intracellular guanosine 3′, 5′-cyclic monophosphate (cGMP) through two subtypes of particulate guanylyl cyclase (GC), GC-A for ANP and BNP, and GC-B for CNP [2]. In skeletal tissues, we have demonstrated that CNP is a positive growth regulator of long bones formed through endochondral ossification via the GC-B/cGMP pathway [3,4]. CNP-depleted mice are characterized by short stature with a phenotype histologically similar that of achondroplasia [3], while the growth plates of explanted long bones in the presence of CNP show a similar histological picture to that of the growth plate cartilage of fibroblast growth factor receptor 3 (FGFR-3)-depleted mice [5]. This raises the possibility that activation of the CNP/GC-B pathway of endochondral bone regulation reverses the inhibitory effect of FGFR-3 signaling in skeletogenesis.

FGFR-3 belongs to a class of tyrosine kinase receptors involved in signal transduction. In the presence of soluble or

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cell-surface heparin sulfate proteoglycans, fibroblast growth factors (FGFs) binding to FGFRs induce receptor dimerization and autophosphorylation on tyrosine residues. This then triggers cell proliferation or differentiation through the Ras-Raf-dependent and phospholipase C-dependent signal transduction pathways involving MAPK stimulation [6]. FGFR-3 mutations have been shown to be responsible for achondroplasia, hypochondroplasia and thanatophoric dysphasia (TDI and TDII) [7,8]. These mutations activate receptor signaling by either inducing ligand-independent receptor dimerization or easing the constraints on autophosphorylation of receptor-tyrosine kinase [9]. It has recently been reported that, of the 23 members of the FGF family, FGF-18 is a physiologic ligand for FGFR-3 in chondrocytes and plays an important role as a mediator in skeletal development [10–12].

To gain further insight into the cellular basis of the interaction between CNP and FGFs in endochondral bone formation, we used ATDC5 cells, which constitute a mouse chondrogenic cell line derived from embryogenic carcinoma cells [13]. In the presence of insulin, these cells differentiate into chondrocytes, form cartilage nodules, serially exhibit several differentiation markers for the chondrocytes, and are eventually mineralized, thus reflecting the endochondral ossification process in vivo. We previously demonstrated that ATDC5 cells contain particularly high activity levels for GC-B and also appear to contain low levels of GC-A and the soluble form of guanylyl cyclase, which is responsive to nitric oxide [14]. Therefore, ATDC5 cells are considered to be a good model to study the interaction between CNP and FGFs in vitro.

We also studied the effects of CNP and FGF18 on organ-cultured fetal mouse tibias. Since the growth plates consist of several zones, each representing a different stage of differentiation and functioning differently, the interaction between the cells in the different zones can be crucial for a given substance to exert its effects. We previously developed an ex vivo organ culture system of mouse long bones [4]. During a 5-day culture, the bones exhibited longitudinal growth mostly due to the growth in the cartilage primordial rather than the ossified portion. This system was therefore considered a good ex vivo model for studying the interaction between CNP and FGFs. The purpose of the study presented here was to clarify the interaction between the CNP/GC-B pathway and FGF signaling in growth plate chondrocytes, as well as the mechanism of this interaction, in order to determine the efficacy of activation of CNP/GC-B as a novel therapeutic strategy for achondroplasia.

Materials and methods

Human C-type natriuretic peptide was purchased from Peptide Institute, Inc. (Minoh, Japan), 8-bromo cGMP and isobutylmethylxanthine (IBMX) from Sigma-Aldrich Co. (St. Louis, MO, USA), and human recombinant FGF2 from Pepro Tech EC. Ltd. (London, England). Rat recombinant FGF18 was generously provided by Amgen Inc. (Thousand Oaks, CA, USA). Primary antibodies, rabbit anti-phospho-ERK1/2 antibodies, rabbit anti- ERK1/2 antibodies, and anti-STAT-1 antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA), rabbit anti-phospho-STAT-1 antibodies from Upstate Biotechnology, (Lake Placid, NY, USA), and HRP-conjugated donkey anti-rabbit IgG antibodies from Amersham Pharmacia Biotech, (Freiburg, Germany). The MEK (MAPK-ERK kinase) inhibitors U0126 and PD098059 were purchased from Cell Signaling Technology Inc., fetal calf serum (FCS) was purchased from Sankou Junyaku (Tokyo, Japan), and Ham F12/DMEM 50/50 medium and Bigger's BJG medium were obtained from GIBCO (Grand Island, NY, USA).

Cell culture conditions

Cells were grown and maintained using standard techniques. ATDC5 cells were maintained in Ham F12/DMEM 50/50 medium containing 5% FCS, antibiotics, and insulin (10 ng/ml). Confluent cells were maintained for 14 days and considered quiescent after maintenance in 0.5% FCS for 24 h. For radioimmunoassay, cells were seeded on 24-multiwell culture plates, and on a 6 cm dish (BD Bioscience, NJ, USA) for Western blotting and real-time PCR analysis.

Intracellular cGMP determination

Quiescent cells were treated with FGFs for 1 h. The cells were then preincubated in Ham F12/DMEM 50/50 medium containing 0.5% FCS and 1 mM IBMX at room temperature for 10 min. CNP was added at a concentration of 10^{-9}–10^{-7} M and incubated at 37°C for 30 min in the presence of 1 mM IBMX. Reactions were terminated immediately by aspirating the medium, washing the cells with ice-cold PBS, and freezing them in 500 μl of 50 mM HCl. The acidified extracts were analyzed for guanylyl cyclase activity. The level of cGMP was determined by radioimmunoassay after succinylation (Yamasa Co. Ltd., Chosi, Chiba, Japan). To examine the effect of FGFs, we also used the MEK inhibitors U0126 and PD098059, which were added 1 h before treatment of CNP. The level of cGMP was determined as already described.

Western blot analysis

Quiescent cells were incubated with a medium containing CNP (10^{-7}–10^{-6} M) or 10^{-4} M 8-bromo cGMP for 1 h. The medium was then switched to an FGF-containing one, and the cells were treated with FGFs (10 ng/ml) for 3 min. Cells were extracted with the aid of a solvent solution (0.5 M Tris–HCl, 10% SDS, β-mercaptoethanol, glycerol, and Bromo-phenol blue). Soluble proteins were electrophoretically resolved on 8% acrylamide, 0.1% SDS gels and

transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). The membranes were probed overnight with antibodies against phosphorylated ERK1/2, ERK1/2, phosphorylated STAT-1 or STAT-1, according to the supplier's instructions. The membranes were then probed with secondary antibodies for 1 h. Bound antibodies were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and their density measured by using the public domain National Institute of Health IMAGE program.

The expression of GC-B in ATDC5 cells was analyzed by Western blot analysis. Quiescent cells were incubated with a medium containing FGF18 (1 or 10 ng/ml) for 1 h, after which the cells were extracted and the proteins blotted to the membrane as described above. The membranes were probed for 1 h with the rabbit polyclonal anti-GC-B antibody [15] (a generous gift from Dr. D.L. Garbers of the University of Texas Southwestern Medical Center) and bound antibodies were detected as described above.

To confirm the effect of the MEK inhibitor on phosphorylated ERK1/2, we used 20 μM U0126, and the MEK inhibitor (U0126) was added 1 h before treatment of FGF2.

Real-time PCR analysis of FGFR-3

Quiescent cells were incubated with a medium containing CNP (10^{-6} M) for 1 h and total RNAs were extracted using ISOGEN (Nippon Gene Co. Ltd., Toyama, Japan) according to the manufacturer's instructions. After synthesis of the first-strand cDNA from 1 μg of total RNA by means of Superscript II RT (Life Technologies, Inc., St. Louis, MO, USA) with random hexamers, Taqman-PCR was performed with the ABI Prism 7700 sequence detection system and Taqman Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA) using FAM and VIC-labeled fluorogenic probes specific for FGFR-3 or the internal standard 18 S rRNA. All samples were run in duplicate in 96-well plates in the ABI Prism 7700 sequence. There was no significant difference in 18 S rRNA levels among experimental groups.

Organ culture of embryonic mouse tibias

Organ culture of fetal mouse tibias was performed with the suspension culture technique in a chemically defined medium (Bigger's BJG medium). Tibial explants from 16-day-old normal ICR mouse embryos were cultured for 5 days with or without 10 ng/ml FGF18 and 10^{-7} M CNP. After a 5-day culture, the total bone length was measured longitudinally by using a linear ocular scale mounted on an inverted microscope. Explants were fixed in 4% paraformaldehyde, decalcified in 10% EDTA/0.1 M Tris–HCl, pH 7.4, for 7 days, and embedded in paraffin. 5-μm-thick sections cut from the paraffin-embedded specimens were stained with Alcian blue (pH 2.5) and hematoxylin/eosin (H&E).

Immunohistochemical staining for type X collagen, using a polyclonal rabbit anti-type X collagen antibody (1:5000; LSL, Tokyo, Japan) as a primary antibody, was also performed. Immunoreactions were visualized by using a biotinylated antipolyvalent antibody, a streptavidin–biotin–horseradish peroxidase complex, and diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA, USA). The specificity of the immunoreactions was controlled by omitting the primary antibody.

The size of hypertrophic cells was measured on 5-μm-thick sections of cultured tibias with a computerized measurement system (KS400 Imaging System; Carl Zeiss, Eching, Germany), and the cells of hypertrophic chondrocytes were manually counted.

Statistical analysis

Data are expressed as the mean ± SE. The changes in cGMP were compared by means of ANOVA using Fisher's test. Comparisons between groups of organ cultured bone lengths were performed with the unpaired t test. Probabilities less than 0.05 were considered statistically significant.

Results

Inhibition by FGF2 of CNP signaling in ATDC5 cells

ATDC5 cells were differentiated into chondrocytes for a 14-day culture after confluency [13], and we confirmed that collagen type X, a marker of hypertrophic chondrocytes, was expressed in these cells [16]. In ATDC5 cells, CNP (10^{-9}–10^{-7} M) stimulated the production of intracellular cGMP in a dose-dependent manner with a 36-fold increase of the basal level at 10^{-7} M CNP (55 ± 3 fmol/well to 1985 ± 181 fmol/well). This increase was inhibited up to 53% by the addition of 10 ng/ml of FGF2 (1045 ± 65 fmol/well) (Fig. 1A). Pretreatment with U0126, a specific inhibitor of the upstream of ERK (MEK) [17], at a concentration of 20 μM partially undid the reduction of cGMP by FGF2 (1395 ± 144 fmol/well vs. 1980 ± 143 fmol/well) compared with the vehicle (Fig. 1B). The same level of recovery was observed after pretreatment with another MEK inhibitor, PD098059, also at a concentration of 20 μM (1425 ± 276 fmol/well vs. 1620 ± 206 fmol/well). Western blot analysis was performed to confirm the blockade of U0126 to ERK1/2 phosphorylation by FGF2. Pretreatment with 20 μM U0126 completely blocked the phosphorylation of ERK1/2 (Fig. 1B inset).

Inhibition by FGF18 of CNP signaling in ATDC5 cells

We analyzed the effect of FGF18, the specific ligand for FGFR-3 in chondrocytes, on CNP-dependent cGMP production in ATDC5 cells. As in the case of FGF2, FGF18 inhibited intracellular cGMP production in ATDC5 cells in a dose-dependent manner (0.3–100 ng/ml). Preincubation

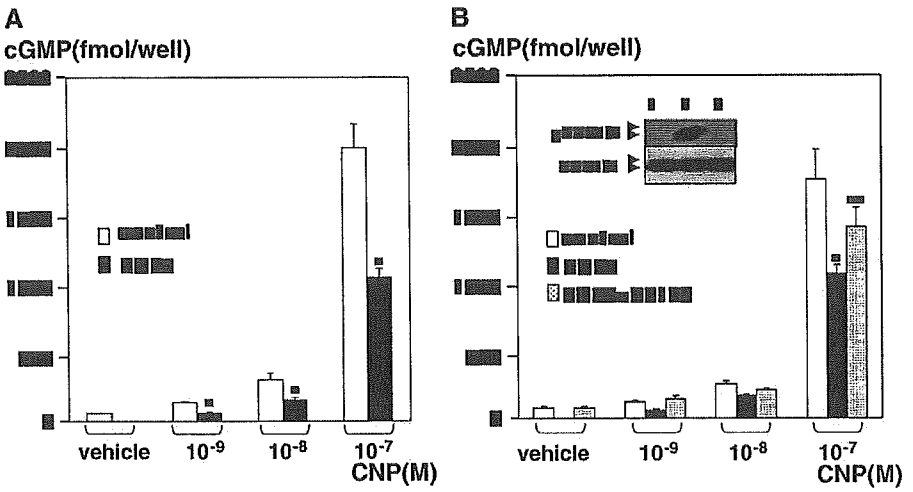

Fig. 1. Inhibition by FGF2 of CNP signaling in ATDC5 cells. The effects of FGF2 on CNP-dependent cGMP production in ATDC5 cells. (A) Controls (open columns) show CNP stimulation of the intracellular cGMP production in a dose-dependent manner (10^{-9}–10^{-7} M CNP), and this increase was inhibited by pretreatment with 10 ng/ml FGF2 (closed columns). Columns represent means ± SE (n = 6, each), $^{*}P < 0.01$ vs. control (B) MEK inhibitor, U0126 (dotted columns) resulted in recovery from the reduction of cGMP by 10 ng/ml FGF2 (closed columns). Columns represent means ± SE (n = 6, each), $^{*}P < 0.01$ vs. control (DMSO 0.0025%) (open columns), $^{**}P < 0.01$ vs. FGF2. Inset, 20 μM U0126 completely blocked the phosphorylation of ERK1/2 in ATDC5 cells. 1: Control (DMSO 0.0025%), 2: treatment with 10 ng/ml FGF2, 3: pretreatment with 20 μM U0126.

with 3 ng/ml FGF18 significantly inhibited the increase in 10^{-7} M CNP stimulated cGMP production (1425 ± 82 fmol/well vs. 1975 ± 111 fmol/well). This inhibition by FGF18 reached 64% at a concentration of 10 ng/ml and 100 ng/ml FGF18 (1255 ± 11 fmol/well) (Fig. 2).

No effect of FGF18 on GC-B expression in ATDC5 cells

To confirm the expression level of GC-B, the specific receptor for CNP in ATDC5 cells, we performed Western blot analysis using antiserum specific for GC-B. The quiescent ATDC5 cells expressed a certain amount of GC-B at 120-kDa band, which incubation with FGF18 (1 or 10 ng/ml) for 1 h did not alter (Fig. 2 inset).

Attenuation by CNP of MAPK activity of FGFs

The effect of CNP on the downstream signaling of FGFR-3 in ATDC5 cells was analyzed next. ERK1/2 phosphorylation was barely detectable at the basal level but was noticeably stimulated with the addition of FGF2 (10 ng/ml) and FGF18 (10 ng/ml) in ATDC5 cells. Treatment of quiescent ATDC5 cells with CNP (10^{-7}–10^{-6} M) for 1 h prior to the addition of FGF2 and FGF18 reduced the phosphorylation of ERK1/2 in a dose-dependent manner. 10^{-6} M CNP completely eliminated the FGF-stimulated phosphorylation of ERK1/2 (Figs. 3A, B), while 10^{-4} M 8-bromo cGMP also inhibited ERK1/2 phosphorylation of FGF2 (Fig. 3C).

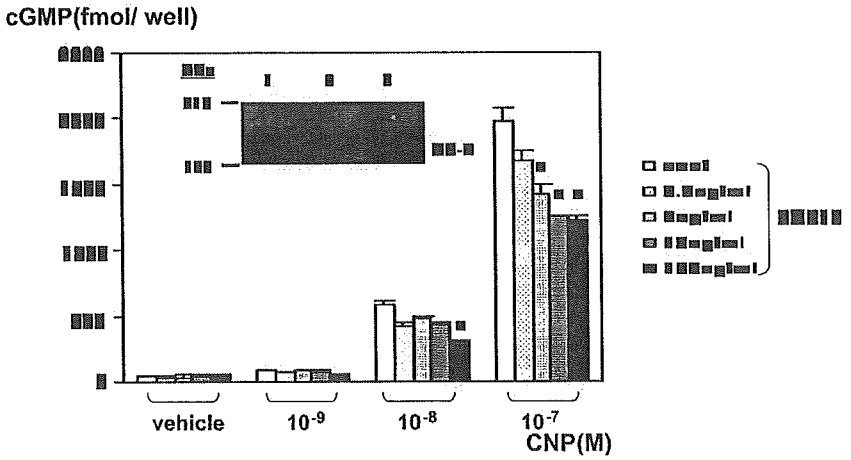

Fig. 2. Inhibition by FGF18 of CNP signaling in ATDC5 cells. The effects of FGF18 on CNP-dependent cGMP production in ATDC5 cells. As in the case of FGF2, FGF18 inhibited the increase in CNP induced cGMP in a dose-dependent manner (0.3–100 ng/ml). Columns represent means ± SE (n = 6, each), $^{*}P < 0.01$ vs. control. Inset, expression of GC-B in ATDC5 cells (Western blot analysis). 1: Vehicle, 2: treatment with 1 ng/ml FGF18, 3: treatment with 10 ng/ml FGF18.

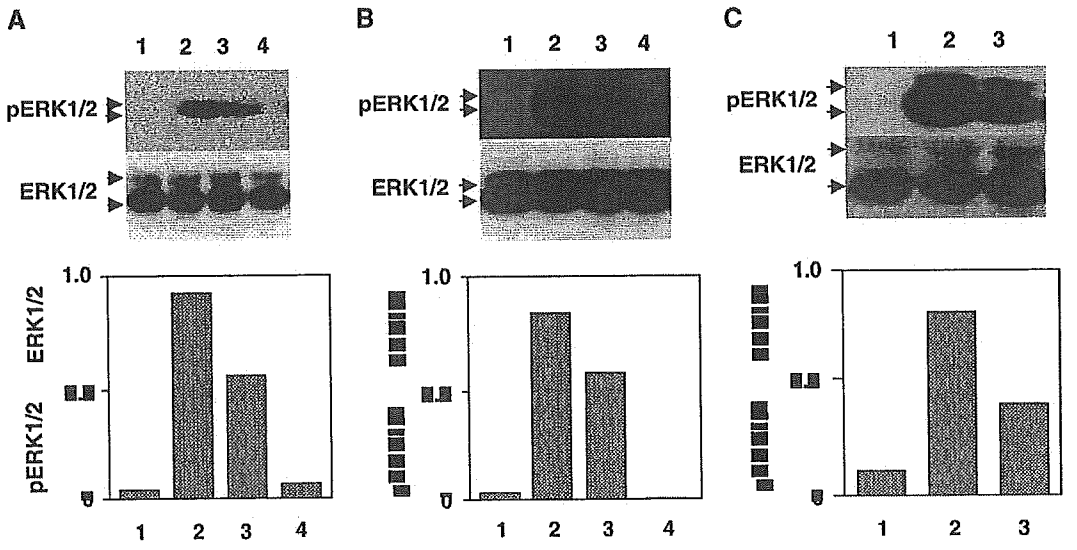

Fig. 3. Attenuation by CNP of MAPK activity of FGFs. CNP and cGMP reduced FGF-stimulated elevation of phosphorylated ERK1/2. ATDC5 cell extracts were examined with Western blotting for phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 (ERK1/2) as described under Materials and methods. (A) CNP diminished FGF2-stimulated elevation of phosphorylated ERK1/2. 1: Vehicle, 2, 3, 4: stimulation with 10 ng/ml FGF2, 3: preincubation with 10^{-7} M CNP, 4: preincubation with 10^{-6} M CNP. (B) CNP attenuated FGF18-stimulated elevation of phosphorylated ERK1/2. 1: Vehicle, 2, 3, 4: stimulation with 10 ng/ml FGF18, 3: preincubation with 10^{-7} M CNP, 4: preincubation with 10^{-6} M CNP. (C) 8-bromo cGMP decreased FGF2-stimulated elevation of phosphorylated ERK1/2. 1: Vehicle, 2, 3: stimulation with 10 ng/ml FGF2, 3: preincubation with 10^{-4} M 8-Bromo cGMP. Representative blots are shown, and the relative levels of proteins were measured as phospho-ERK1/2/ERK1/2 density values. Each experiment was repeated 3 times, and data from a representative experiment are shown.

No change in STAT-1 phosphorylation in ATDC5 cells by FGFs or CNP

STAT-1 phosphorylation was already detectable at the basal level in ATDC5 cells. The level of phosphorylated STAT-1 remained unchanged when treated with FGF2 or FGF18 for 3, 10, 15, 30, or 60 min. Treatment of quiescent ATDC5 cells with CNP (10^{-7}–10^{-6} M) prior to the addition of FGF2 (10 ng/ml) and FGF18 (10 ng/ml) did not alter the amount of phosphorylated STAT-1 (Figs. 4A,

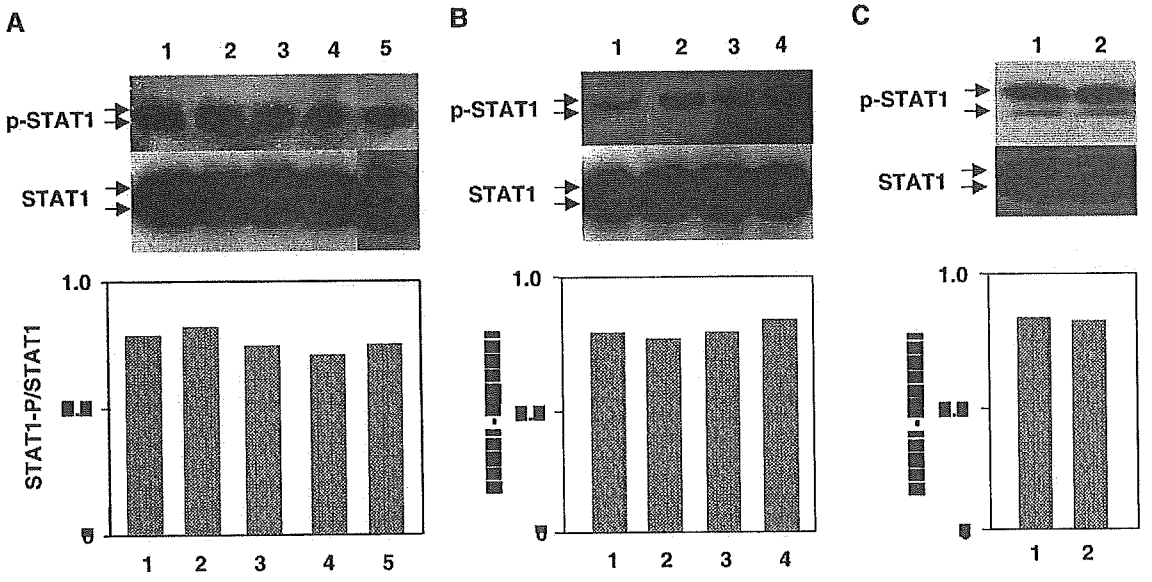

Fig. 4. No change in STAT-1 phosphorylation in ATDC5 cells by FGFs or CNP. Effects of CNP on the STAT-1 pathways of FGFs. ATDC5 cell extracts were examined with Western blotting for phosphorylated STAT-1 (pSTAT-1) and total STAT-1 (STAT-1) as described under Materials and methods. (A) CNP and 8-bromo cGMP did not alter the level of phosphorylated STAT-1 after FGF2 stimulation. 1: Vehicle, 2, 3, 4, 5: stimulation with 10 ng/ml FGF2, 3: preincubation with 10^{-7} M CNP, 4: preincubation with 10^{-6} M CNP, 5: preincubation with 10^{-4} M 8-bromo cGMP. (B) CNP did not alter the level of phosphorylated STAT-1 after FGF18 stimulation. 1: Vehicle, 2 3, 4: stimulation with 10 ng/ml FGF18, 3: preincubation with 10^{-7} M CNP, 4: preincubation with 10^{-6} M CNP. (C) CNP did not alter the basal expression of phosphorylated STAT-1. 1: Vehicle, 2: 10^{-6} M CNP alone. Representative blots are shown, and the relative levels of proteins were measured as the phospho-STAT-1/STAT-1 density values. Each experiment was repeated 3 times, and the data from a representative experiment are shown.

B), nor did CNP itself (10^{-6} M) affect the basal level of phosphorylated STAT-1 in ATDC5 cells (Fig. 4C), or treatment of quiescent ATDC5 cells with 10^{-4} M 8-bromo cGMP prior to the addition of FGF2 (10 ng/ml) cause no change in the amount of phosphorylated STAT-1 (Fig. 4A).

No effect of CNP on FGFR-3 expression in ATDC5 cells

FGFR3 mRNA levels in ATDC5 cells treated with the vehicle and pretreated with CNP (10^{-6} M) were 1.00 ± 0.06 and 1.04 ± 0.12 in arbitrary units, respectively, and this difference was not significant.

Effect of CNP and FGF18 on mouse fetal tibia organ culture

The organ culture of fetal mouse tibias provides a unique in vitro experimental model system of endochondral ossification. For our study, we used cultured tibias prepared from ICR mice, to examine the effects of CNP and FGF18. Treatment with 10^{-7} M CNP for 5 days produced a 12% increase in the total length of tibial explants compared with vehicle-treated explants (vehicle treated: 3.56 ± 0.05 mm vs. CNP treated: 3.99 ± 0.07 mm). On the other hand, treatment with 10 ng/ml FGF18 for 5 days resulted in a 6% decrease in the total length of tibial explants compared with vehicle-treated explants (vehicle treated: 3.57 ± 0.03 mm vs. FGF18 treated: 3.37 ± 0.04 mm). Treatment with a combination of 10^{-7} M CNP and 10 ng/ml FGF18 increased the total length of tibial explants for 8% compared with vehicle-treated explants (vehicle treated: 3.55 ± 0.04 mm vs. CNP and FGF18 treated: 3.84 ± 0.06 mm). These differences were all statistically significant ($P < 0.01$) (Fig. 5).

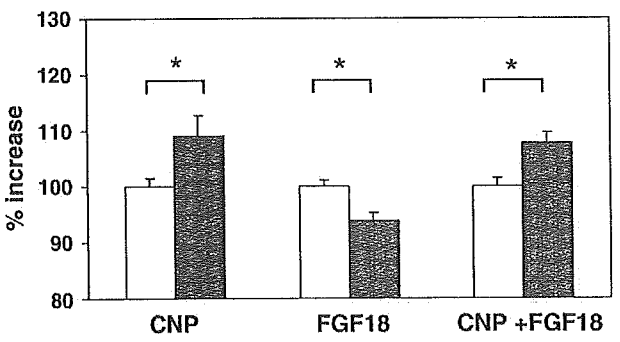

Fig. 5. Effect of CNP and FGF18 on mouse fetal tibia organ culture. Percent change in total length of fetal mouse tibias cultured with CNP and/or FGF18 for 5 days. Open columns show the percent change in total length of one tibia from mouse fetus treated with the vehicle. Hatched columns show the percent change in total length of the other tibia from the same mouse treated with 10^{-7} M CNP, 10 ng/ml FGF18, or with a combination of 10^{-7} M CNP and 10 ng/ml FGF18. Columns represent means ± SE ($n = 5$, each), *$P < 0.01$ vs. vehicle.

Effect of CNP and FGF18 on cell size and cell numbers of the growth plate chondrocytes

Microscopic examination disclosed elongation of the growth plate in mouse tibias cultured with 10^{-7} M CNP. Higher magnification of Alcian blue HE staining (Figs. 6A, B, E, F) and immunohistochemical staining for type X collagen (Figs. 6I, J) showed that the hypertrophic chondrocyte layer increased after treatment with 10^{-7} M CNP compared with treatment with the vehicle. The mean size of hypertrophic chondrocytes was markedly increased (vehicle treated: 497.85 ± 19.2 vs. CNP treated: 1071.42 ± 53.5 μm^2) (Fig. 7A), and the number of cells in the hypertrophic chondrocyte layer was reduced (vehicle treated: 152.67 ± 4.1 vs. CNP treated: 118 ± 3.61 cells) (Fig. 7B). In contrast, culturing with 10 ng/ml FGF18 caused shortening of the growth plate in cultured mouse tibias compared with that in vehicle-treated ones. Higher magnification of Alcian blue HE staining (Figs. 6A, C, E, G) and immunohistochemical staining for type X collagen (Figs. 6I, K) demonstrated that the hypertrophic chondrocyte layer was reduced. Not only the cell size (vehicle treated: 497.85 ± 19.2 vs. FGF18 treated: 314.01 ± 23.67 μm^2) (Fig. 7A), but also the number of hypertrophic chondrocytes was reduced by the treatment with 10 ng/ml FGF18 (vehicle treated: 152.67 ± 4.1 vs. FGF18 treated: 89.3 ± 1.45 cells) (Fig. 7B). Culturing with both 10^{-7} M CNP and 10 ng/ml FGF18 resulted in recovery by CNP of shortening of the growth plate in cultured mouse tibias by FGF18. Higher magnification of Alcian blue HE staining (Figs. 6C, D, G, H) and immunohistochemical staining for type X collagen (Figs. 6K, L) showed an increase in the hypertrophic chondrocyte layer compared with that of the FGF18-treated ones. The mean size of hypertrophic chondrocytes also increased compared with that of FGF18-treated ones (FGF18 treated: 314.01 ± 23.67 vs. CNP and FGF18 treated: 751.16 ± 41.6 μm^2) (Fig. 7A), and the reduction in number was also undone by CNP (FGF18 treated: 89.3 ± 1.45 vs. CNP and FGF18 treated: 145.3 ± 4.41 cells) (Fig. 7B).

Discussion

The study reported here used two different experimental designs to examine the interaction of the CNP/GC-B and FGFR-3 pathways in mouse chondrogenic ATDC5 cells and in organ-cultured tibias. In this study we were able to show that: (1) ATDC5 cells express GC-B, (2) FGF2 and FGF18 reduce CNP-dependent cGMP production in a dose-dependent manner without changing the amount of GC-B, (3) MAPK inhibitors attenuate the FGF inhibition of CNP-dependent cGMP production, (4) both CNP and cGMP inhibit the MAPK pathway but not the STAT-1 pathway of FGFR-3 activation without changing the amount of FGFR-3, and (5) CNP and FGF18 counteract longitudinal bone growth in organ cultured tibias.

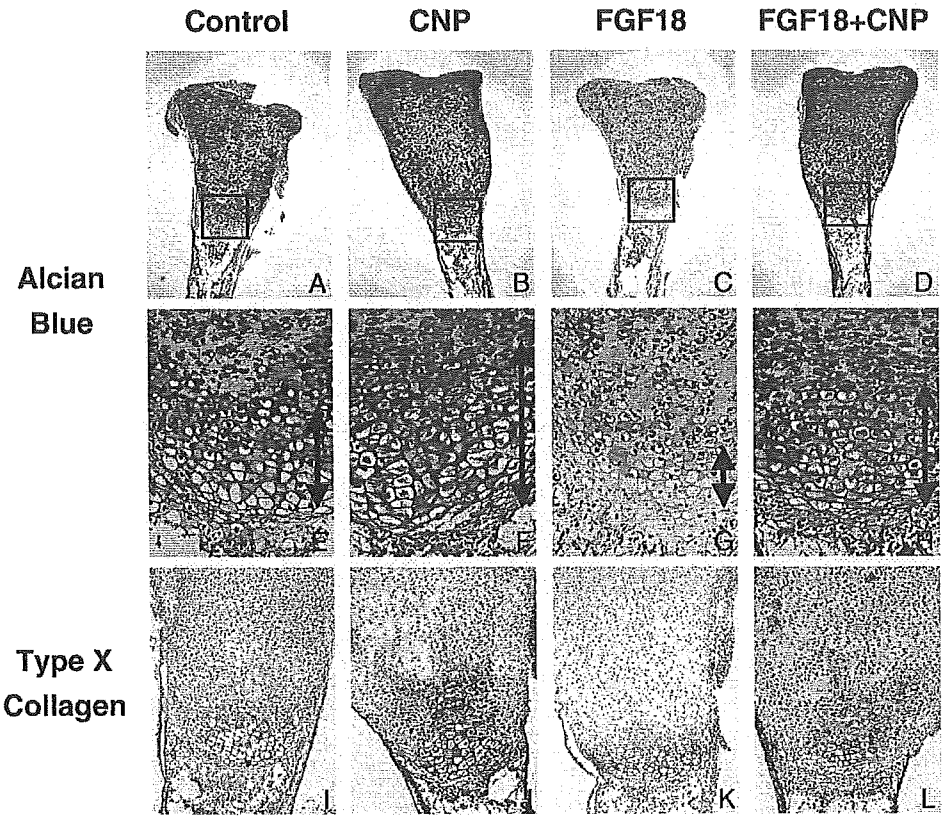

Fig. 6. Effect of CNP and FGF18 on mouse fetal tibia organ culture. Alcian blue and hematoxylin–eosin staining (upper and middle panels), and immunohistochemical staining for type X collagen (bottom panel) of cultured tibiae with CNP and/or FGF18. A, E, I: control; B, F, J: 10^{-7} M CNP; C, G, K: 10 ng/ml FGF18; D, H, L: 10 ng/ml FGF18 and 10^{-7} M CNP. Arrows indicate the hypertrophic chondrocyte zone. (A–D: magnification ×4, E–H: magnification ×20, I–L: magnification ×10.)

Substantial evidence exists that CNP is an antagonist of mitogenic action in many cell types. Activation of the CNP/GC-B pathway in the vascular smooth muscle cells was found to attenuate the onset of DNA synthesis, diminish cell proliferation, and inhibit chemotaxis [18]. As for the interaction of CNP with FGF signaling, marked elevation of cGMP induced by CNP had been reported to block the activation of the MAPK cascade induced by FGFs in fibroblast [19]. These indicate that significant antagonistic interplay may also occur between the CNP/GC-B pathway and growth factor-regulated pathways in growth plate chondrocytes.

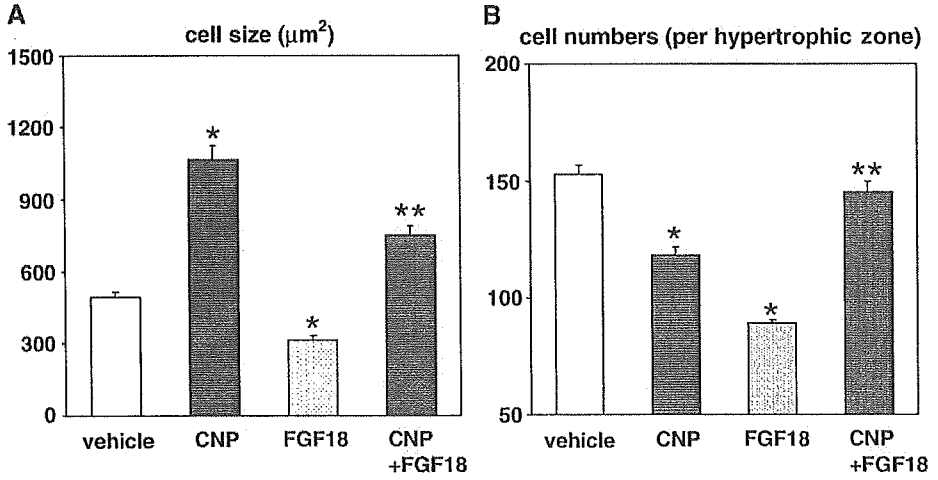

Fig. 7. Effect of CNP and FGF18 on mouse fetal tibia organ culture. (A) Effect of CNP and FGF18 on the size of hypertrophic chondrocytes (n = 10). Columns represent means ± SE (n = 10, each), $^{*}P < 0.01$ vs. vehicle, $^{**}P < 0.01$ vs. FGF18. (B) Effect of CNP and FGF18 on the numbers of hypertrophic chondrocytes (n = 5). Columns represent means ± SE (n = 5, each), $^{*}P < 0.01$ vs. vehicle, $^{**}P < 0.01$ vs. FGF18.

Constitutive activation of FGFR-3 has been reported to inhibit the proliferation and differentiation of the growth plate chondrocytes [20]. Our study clarified that CNP can undo the reduction in the size and numbers of chondrocytes of the hypertrophic zone of the growth plate of mouse tibias expressing the type X collagen, and compensate for the shortening of the growth plate resulting from the treatment with FGF18. These results are consistent with the findings of previous in situ hybridization studies which have shown that the mouse *fgfr-3* gene is widely expressed in the proliferative and prehypertrophic chondrocyte zones of cartilage [20], thus, overlapping the CNP/GC-B expression described in a previous study of ours [3]. Therefore, the interaction between the CNP/GC-B and FGFR-3 pathways in these cells results in the change of the hypertrophic chondrocytes during the endochondral ossification in the growth plate.

These findings, together with the fact that the specific MAPK inhibitors eliminated the FGF2-inhibition of CNP-dependent cGMP production in ATDC5 cells, suggest that the CNP/GC-B pathway and MAPK pathway counteracts through FGFR-3 in the regulation of growth plate chondrocytes.

Alternatively, one can hypothesize that the MAPK pathway is not the only FGFR-3 signaling pathway in chondrocytes. It has been suggested that the inhibitory effects of FGFs on bone growth are mediated by the STAT-1 pathway of FGFR-3 signaling [21], while a highly controlled balance between the MAPK and STAT-1 pathways has been demonstrated recently in growth factor-stimulated cells. The observation that the *Stat-1* null mice with overexpressing FGF2 can overcome apoptosis and the reduction in chondrocyte proliferation gives support to the view that the Stat-1 pathway may also have a key function in growth retardation [22]. In keeping with these findings, however, our observation of a significant ligand-independent STAT-1 phosphorylation in ATDC5 cells and no change in the level of STAT-1 phosphorylation as a result of stimulation by FGFs, CNP, or cGMP, leads us to conclude that the mechanism by which CNP undoes the shortening of bone length treated by FGFs consists of overcoming the MAPK-mediated pathway, not the STAT-1-mediated pathway.

Constitutive activation of ERK1 in chondrocytes reportedly induces a condition resembling achondroplasia [23]. Many studies have shown that activating mutations in FGFR-3 inhibit bone growth in patients with achondroplasia and thanatophoric dysplasia [24,25], while overexpression of FGF2 in mice slows longitudinal growth [26] and inactivating knockout mutations in FGFR-3 increase longitudinal long bone growth in mice [5]. On the other hand, CNP null mice as well as GC-B null mice are characterized by short stature with a phenotype histologically similar to that of achondroplasia [27]. Recently, we have shown that achondroplasia model mice, expressing the constitutive active mutant form of FGFR-3 (G380R) in cartilage [5], recovered from abnormal growth plate development and dwarfing phenotype as a result of the overexpression of CNP in their cartilage [28], suggesting that interaction between the CNP/GC-B and FGFR-3 pathways also occurs in vivo. Although two pathways are antagonistic, the effect of the CNP/GC-B pathway can overcome the effect of FGFR-3 pathway, partly because the activation level of downstream signaling of FGFR-3 (G380R) mutant is relatively weak [26], compared to the overexpressed CNP. During the course of this study, mutations in the human GC-B gene were reported to cause acromesomelic dysplasia, type Maroteaus, a type of skeletal dysplasia [29]. Therefore, the CNP/GC-B pathway appears critical for the proper progression of endochondral ossification also in human bone.

The downstream of the interaction between CNP/GC-B/cGMP pathway and FGFR-3/MAPK pathway remains unexplored. We recently reported that cGMP-dependent kinase depleted mice ($Prkg2^{-/-}$) showed abnormal growth plate development and dwarfing phenotype, that these changes were not affected by the overexpression of CNP in cartilage [30] and that the growth plate chondrocyte differentiation was disorganized, which is different from what has been observed in CNP-depleted mice.

GC-B is constitutively phosphorylated while receptor phosphorylation is absolutely essential for hormonal activation. On the other hand, the dephosphorylation of GC-B in response to hormone binding has been shown to correlate with the declining activity of these receptors in whole cells, suggesting that receptor dephosphorylation mediates the homologous desensitization of the receptor [31]. As the expression level of GC-B was not changed by FGF18 stimulation in our study, this dephosphorylation process may be involved in the desensitization of GC-B after FGFR-3 stimulation.

The clearance receptor of the natriuretic peptide reportedly mediates antimitogenic action of CNP in some but not all cell lines [32]. To determine if the effects of CNP were mediated by the clearance receptor in the ATDC5 cells, C-ANF, a selective ligand for the clearance receptor, was tested on ATDC5 cells and did not inhibit basal or FGF-stimulated ERK1/2 phosphorylation (data not shown).

We have demonstrated that the CNP/GC-B pathway engages in negative cross talks with FGFR pathways, that FGFs reduce CNP-dependent intracellular cGMP production and that CNP and cGMP markedly diminish the FGF-induced phosphorylation of ERK1/2 in chondrocytes. We also showed that CNP does not affect the amount of phosphorylated STAT-1 in chondrocytes. The results of our study show that FGFs and the activity of MAPK play an important role in the growth of chondrocytes, and negatively interact with the CNP/GC-B pathway and explain one of the molecular mechanisms of the growth stimulating action of CNP, suggesting that activation of the CNP/GC-B pathway may be effective for the treatment of achondroplasia.

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References

[1] Nakao K, Ogawa Y, Suga S, Imura H. Molecular biology and biochemistry of the natriuretic peptide system: I. natriuretic peptides. J Hypertens 1992;10:907–12.
[2] Nakao K, Ogawa Y, Suga S, Imura H. Molecular biology and biochemistry of the natriuretic peptide system: II. natriuretic peptide receptors. J Hypertens 1992;10:1111–4.
[3] Chusho H, Tamura N, Ogawa Y, Yasoda A, Suda M, Miyazawa T, et al. Dwarfism and early death in mice lacking C-type natriuretic peptide. Proc Natl Acad Sci USA 2001;98:4016–21.
[4] Yasoda A, Ogawa Y, Suda M, Tamura N, Mori K, Sakuma Y, et al. Natriuretic peptide regulation of endochondral ossification. Evidence for possible roles of the C-type natriuretic peptide/guanylyl cyclase-B pathway. J Biol Chem 1998;273:11695–700.
[5] Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat Genet 1996;4:390–7.
[6] Kanai M, Goke M, Tsunekawa S, Podolsky DK. Signal transduction pathway of human fibroblast growth factor receptor 3. Identification of a novel 66-kDa phosphoprotein. J Biol Chem 1997;272:6621–8.
[7] Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell 1996;84:911–21.
[8] Tavormina PL, Rimoin DL, Thompson LM, Zhu YZ, Wilkin DJ, Lachman RS, et al. Thanatophoric dysplasia (type I and II) caused by distinct mutations in fibroblast growth factor receptor 3. Nat Genet 1995;9:321–8.
[9] Shimizu A, Tada K, Shukunami C, Hiraki Y, Kurokawa T, Magane N, et al. A novel alternatively spliced fibroblast growth factor receptor 3 isoform lacking the acid box domain is expressed during chondrogenic differentiation of ATDC5 cells. J Biol Chem 2001;276: 11031–40.
[10] Ellsworth JL, Berry J, Bukowski T, Claus J, Feldhaus A, Holderman S, et al. Fibroblast growth factor-18 is a trophic factor for mature chondrocytes and their progenitors. Osteoarthr Cartil 2002;10:308–20.
[11] Ohbayashi N, Shibayama M, Kurotaki Y, Imanishi M, Fujimori T, Itoh N, et al. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. Genes Dev 2002; 16:870–9.
[12] Liu Z, Xu J, Colvin JS, Ornitz DM. Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. Genes Dev 2002; 16:859–69.
[13] Shukunami C, Shigeno C, Atsumi T, Ishizeki K, Suzuki F, Hiraki Y. Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. J Cell Biol 1996;133:457–68.
[14] Suda M, Tanaka K, Yasoda A, Komatsu Y, Chusho H, Miura M, et al. C-type natriuretic peptide/guanylate cyclase B system in ATDC5 cells, a chondrogenic cell line. J Bone Miner Metab 2002;20:136–41.
[15] Tamura N, Garbers DL. Regulation of the guanylyl cyclase-B receptor by alternative splicing. J Biol Chem 2003;278:48880–9.
[16] Miura M, Tanaka K, Komatsu Y, Suda M, Yasoda A, Sakuma Y, et al. Thyroid hormones promote chondrocyte differentiation in mouse ATDC5 cells and stimulate endochondral ossification in fetal mouse tibias through iodothyronine deiodinases in the growth plate. J Bone Miner Metab 2002;17:443–54.
[17] Hotokezaka H, Sakai E, Kanaoka K, Saito K, Matsuo K, Kitaura H, et al. U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells. J Biol Chem 2002;277:47366–72.
[18] Komatsu Y, Itoh H, Suga S, Ogawa Y, Hama N, Kishimoto I, et al. Regulation of endothelial production of C-type natriuretic peptide in coculture with vascular smooth muscle cells. Role of the vascular natriuretic peptide system in vascular growth inhibition. Circ Res 1996;78:606–14.
[19] Chrisman TD, Garbers DL. Reciprocal antagonism coordinates C-type natriuretic peptide and mitogen-signaling pathways in fibroblasts. J Biol Chem 1999;274:4293–9.
[20] Naski MC, Colvin JS, Coffin JD, Ornitz DM. Repression of hedgehog signaling and BMP4 expression in growth plate cartilage by fibroblast growth factor receptor 3. Development 1998;125:4977–88.
[21] Sahni M, Ambrosetti DC, Mansukhani A, Gertner R, Levy D, Basilico C. FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. Genes Dev 1999; 13:1361–6.
[22] Sahni M, Raz R, Coffin JD, Levy D, Basilico C. STAT1 mediates the increased apoptosis and reduced chondrocyte proliferation in mice overexpressing FGF2. Development 2001;128:2119–29.
[23] Murakami S, Balmes G, McKinney S, Zhang Z, Givol D, de Crombrugghe B. Constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfism and rescues the Fgfr3-deficient mouse phenotype. Genes Dev 2004;18:290–305.
[24] Shiang R, Thompson LM, Zhu YZ, Church DM, Fielder TJ, Bocian M, et al. Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. Cell 1994; 78:335–42.
[25] Webster MK, D'Avis PY, Robertson SC, Donogue DJ. Profound ligand-independent kinase activation of fibroblast receptor 3 by the activation loop mutation responsible for a lethal skeletal dysplasia, thanatophoric dysplasia type II. Mol Cell Biol 1996;16:4081–7.
[26] Coffin JD, Florkiewicz RZ, Neumann J, Mort-Hopkins T, Dorn II GW, Lightfoot P, et al. Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. Mol Biol Cell 1995;6:1861–73.
[27] Tamura N, Doolittle LK, Hammer RE, Shelton JM, Richardson JA, Garbers DL. Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. Proc Natl Acad Sci USA 2004;101:17300–5.
[28] Yasoda A, Komatsu Y, Chusho H, Miyazawa T, Ozasa A, Miura M, et al. Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. Nat Med 2004;10:80–6.
[29] Bartels CF, Bukulmez H, Padayatti P, Rhee DK, van Ravenswaaij-Arts C, Pauli RM, et al. Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. Am J Hum Genet 2004;75: 27–34.
[30] Miyazawa T, Ogawa Y, Chusho H, Yasoda A, Tamura N, Komatsu Y, et al. Cyclic GMP-dependent protein kinase II plays a critical role in C-type natriuretic peptide-mediated endochondral ossification. Endocrinology 2002;143:3604–10.
[31] Abbey SE, Potter LR. Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B. Endocrinology 2003;144:240–6.
[32] Komatsu Y, Itoh H, Suga S, Igaki T, Ogawa Y, Kishimoto I, et al. Regulation of secretion and clearance of C-type natriuretic peptide in the interaction of vascular endothelial cells and smooth muscle cells. J Hypertens 1996;14:585–92.

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ORIGINAL ARTICLE

High-dose glucocorticoid treatment induces rapid loss of trabecular bone mineral density and lean body mass

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Abstract A recent large-scale study revealed that glucocorticoid treatment increased fracture risk, which occurred at a far smaller dose and by a shorter duration than previously thought. To study the underlying mechanism for the increased risk of fracture, we studied the early changes in bone mineral density (BMD) and body composition by dual energy X-ray absorptiometry (DXA) after initiating high-dose glucocorticoid treatment. High-dose glucocorticoid treatment was arbitrarily defined as daily doses of ≥40 mg of a predonisolone equivalent. The 33 patients enrolled in this study had not received glucocorticoid treatment before. Only 2 months of treatment resulted in substantial BMD loss, most markedly in the lumbar spine, followed by the femoral neck and total body, which suggests the preferential trabecular bone loss. Body composition was also greatly affected. Thus, 2-month treatment with glucocorticoid significantly reduced bone mineral content (BMC), lean body mass (LBM) and increased fat mass (FAT). Our results are likely to have some clinical relevance. First, BMD loss occurs quite rapidly after starting glucocorticoid treatment, and patients receiving glucocorticoid treatment should be more carefully monitored for their BMD. Second, LBM, which mainly represents muscle volume, decreases rapidly after initiating glucocorticoid treatment. Decreased LBM might be also responsible for the increased risk of fracture, since falling is a well-known risk factor for fracture, and patients receiving glucocorticoid treatment should also be evaluated for their body composition.

Keywords Body composition · Dual energy X-ray absorptiometery · Glucocorticoid-induced osteoporosis · Lean body mass · Steroid myopathy

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Introduction

Among the various adverse events associated with therapeutic glucocorticoid use, osteoporotic fracture is considered to be the most common complication [1]. A large-scale study from the UK clearly demonstrated that glucocorticoid treatment, even with small dosage and for short duration, significantly increased the risk of fracture [2]. Furthermore, a recent meta-analysis by van Staa et al. showed that the risk of fracture increased quite rapidly (within 3 to 6 months) after initiating the glucocorticoid treatment [3]. Prompted by these works, we have studied the early effects of high-dose glicocorticoid use on the skeletal system.

The purpose of this paper was twofold: first, to examine the initial effects of glucocorticoid on bone mineral density (BMD) at various skeletal sites; second, to study the effects of glucocorticoid on body composition based on the following considerations. Recent evidence suggests that the fracture threshold in patients with glucocorticoid-induced osteoporosis (GIO) is different from that in patients with primary osteoporosis [4]. Although glucocorticoid is known to cause muscle