c-fmsはチロシンキナーゼドメインをもつ受容体である. 一方, RANKにはTRAFs (TNF receptor associated factors) が結合する[10]. TRAFsを介したシグナルは, さらにJNK (c-jun N-terminal kinase), p38MAPK (p38 mitogen-activated protein kinase) およびERK (extracellular signal regulated kinase) などを活性化する. これらのシグナルが破骨細胞の分化を誘導する[11)~13)]. 一方, ノックアウトマウスの解析より, NF-κBとc-Fosは破骨細胞の分化に必須な転写因子であることが示された[14)~17)]. 最近, NFAT2 (nuclear factor of activated T cells, NFATc1) も破骨細胞の分化に重要な転写因子であることが報告された[18)]. 破骨細胞の分化と機能を調節する転写因子の研究は, 今後さらに活発に展開されるであろう.

III 骨形成の調節機構

骨形成を担う骨芽細胞は, 軟骨細胞, 線維芽細胞, 筋芽細胞, 脂肪細胞と同様に未分化間葉系細胞を起源とする (図2). 骨形成を調節する因子としては, PTHやエストロゲンなどのホルモンとTGFβ (transforming growth factor β), FGF (fibroblast growth factor), IGF (insulin-like growth factor), BMPなどのサイトカインが知られる. それらのなかで, BMPは骨芽細胞の分化を最も強力に誘導する因子である. BMPは骨基質中に多量に存在し, 骨折や骨吸収時に放出されパラクリン的に作用すると考えられている. BMPはセリン・スレオニンキナーゼ活性をもつI型およびII型受容体から成るヘテロ二量体と結合する. BMPシグナルは転写因子Smadにより伝達される. BMPが受容体に結合すると, リガンド特異型転写因子Smad1/5/8がリン酸化される. リン酸化されたSmad1/5/8は, 共通型SmadであるSmad4とヘテロ三量体を形成し, 核内に移行し標的遺伝子の転写を促進する. 一方, Smad6/7は抑制型Smadで, I型受容体に結合してリガンド特異型Smadのリン酸化を阻害する. BMPは抑制型Smad6の発現を誘導する. このように, BMPはSmadシグナルのオン/オフをこまめに制御

しながら骨形成を促進する因子である.

Runx2は骨芽細胞の分化に必須な転写因子として発見された. Runx2遺伝子欠損マウスは骨芽細胞の分化に障害があり, 骨が形成されない. また, 頭蓋・肩甲骨の低形成を特徴としたヒトのCleidocranial dysplasia症候群は, Runx2ヘテロ欠損遺伝病であることが知られている[5), 6), 19)]. BMPはRunx2の発現を誘導し, Smad5はRunx2と相互作用して, 骨芽細胞の分化を促進することが報告された[20), 21)]. 一方, Runx2欠損マウスより得た間葉系細胞の骨芽細胞への分化も誘導できる. そのため, BMPはRunx2依存的および非依存的に骨芽細胞の分化を誘導すると考えられる[22)]. 実際に, Osterixは, BMP刺激により骨芽細胞前駆細胞が発現する転写因子として発見された. Osterixノックアウトマウスは軟骨形成とRunx2の発現に異常はないが, Runx2ノックアウトマウスと同様に骨芽細胞が全く存在しない. 一方, Runx2ノックアウトマウスはOsterixも発現しない. そのため, 骨芽細胞の分化において, OsterixはRunx2の下流で働く転写因子と考えられている[23)].

最近, Wntの受容体Frizzledとともにそのシグナルを伝達するLRP5 (low density lipoprotein receptor-related protein 5) の欠損が骨粗鬆症をもたらすことが示された[24), 25)]. さらに, 骨密度に影響を及ぼす遺伝因子を同定する連鎖解析が行われ, LRP5遺伝子上に1アミノ酸置換 (G171V) が見いだされた[26)]. このように, Wntシグナルも骨形成を調節していると考えられ, その詳細な解明が期待される[27)].

IV 骨リモデリング

動物にPTHや1,25(OH)$_2$D$_3$を投与して骨吸収を促進させると, 血中の骨形成マーカーも亢進する. また, 卵巣摘出術を施した動物では, 骨吸収と骨形成が同時に亢進される. このように, 骨吸収と骨形成が共役していることは知られているが, どれほど厳格に共役しているか明らかではなかった. 最近, OPG欠損マウスを用いて, 骨吸収と骨形成がきわめて厳格に共役していることが示された. OPG欠損マウスは, 骨吸収が著しく亢進するため重篤な骨粗鬆

症を呈する．骨形態計測を行ったところ，OPG欠損マウスは骨吸収の亢進とともに，骨形成も著しく亢進していることが判明した．実際に，OPG欠損マウスにおいて，骨形成の指標である血中のアルカリホスファターゼ活性とオステオカルシン値は正常マウスよりも4倍も高値を示す．OPG欠損マウスは骨吸収と骨形成がともに亢進しているが，平衡状態が骨吸収に偏るために骨量が減少する．そこで，OPG欠損マウスに骨吸収抑制薬であるビスフォスフォネートを投与し，骨吸収を抑制したとき，骨形成がどのように制御されるか解析された．OPG欠損マウスにビスフォスフォネートを投与すると骨吸収が著しく抑制され，骨量は増加した．興味深いことに，骨吸収の抑制に伴い，亢進していた骨芽細胞の機能も強力に抑制された．ビスフォスフォネート投与により，OPG欠損マウスの血中のアルカリホスファターゼ活性とオステオカルシン値も正常値に回復した．これらの実験結果は，破骨細胞と骨芽細胞の機能が厳格に共役していることを示すものである．一方，BMPペレットの皮下移植実験より，BMPが誘導する異所性骨形成はOPG欠損マウスと正常マウスの間に差異が認められなかった．この知見は，骨代謝共役を司る因子は液性ではなく局所で作用する因子である可能性を示唆する[28]．これまでに骨代謝異常を示す遺伝子欠損あるいは遺伝子導入された多くのマウスが解析されてきたが，そのほとんどが骨形成と骨吸収がともに増加するか，あるいはともに低下するという傾向が認められる．今後の研究で，骨代謝共役の分子機構の解明が望まれる．

V 骨リモデリング異常としての骨粗鬆症

骨粗鬆症は，骨吸収と骨形成の共役の破綻がもたらした病態と考えられる．老人性の骨粗鬆症は，骨吸収と骨形成がともに低下したリモデリング速度の遅い低回転型骨粗鬆症である．長期的には骨形成の低下が骨吸収の低下よりも大きいため骨量が減少する．骨形成低下の一因に，BMP/Runx2シグナル系やWntシグナル系の活性化低下が関与しているかもしれない．一方，エストロゲン欠乏に起因する閉経

後骨粗鬆症は，骨吸収と骨形成がともに亢進したリモデリング速度の速い高回転型骨粗鬆症である．高回転型骨粗鬆症では，RANKLの誘導が引き金になると考えられる．卵巣摘出動物において，骨局所でIL-1，IL-6，TNF-αなどの骨吸収を促進するサイトカインの産生亢進が認められ[29]～[31]，これらのサイトカインは骨芽細胞のRANKLの発現を誘導する．一方，破骨細胞の機能の亢進は，局所で骨芽細胞の機能を促進する．高回転型骨粗鬆症に認められる骨形成の促進は，この骨代謝共役機構によると考えられる．骨代謝共役の分子機構は解明されていないが，骨吸収抑制薬は，骨形成も同時に抑制することを常に念頭に処方されることが必要であろう．

VI おわりに

骨リモデリングは骨形成と骨吸収の巧みなバランスにより制御される．各種の実験より，骨吸収と骨形成を結ぶ骨代謝共役機構が骨リモデリングの制御の本体であると考えられる．この数年で，破骨細胞と骨芽細胞の分化と機能を調節する因子やシグナルが次々と解明されてきた．今後の研究で，骨代謝共役機構が解明されることが期待される．骨代謝共役の分子機構の解明は，リモデリング速度の遅い低回転型骨粗鬆症とリモデリング速度の早い高回転型骨粗鬆症に適したそれぞれの治療指針の確立にも大いに寄与するであろう．

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抗 RANKL 抗体 AMG 162 による
骨粗鬆症の治療

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RANKL (receoptor activator of NF-κB) は，破骨細胞の分化を誘導するサイトカインである。Amgen 社は RANKL に対するヒト RANKL 抗体 AMG 162 を作製し，骨粗鬆症治療薬としての臨床試験を進めている。49 人の閉経後の女性を対象としたフェーズ I 試験では，AMG 162 単回皮下投与によって，長期間（6 カ月以上）の骨吸収の抑制が持続すること，また重篤の副作用のないことが報告された。さらに，骨量低下を示す閉経後の女性 411 人を対象としたフェーズ II 試験では，AMG 162 単回皮下投与によって 1 カ月以内に骨量が増加することが示された。

A new treatment for osteoporosis using fully human monoclonal antibody to RANKL, AMG 162

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RANKL is an essential factor for osteoclastogenesis. Amgen research group has developed AMG 162, a fully human monoclonal antibody to RANKL. The bone antiresorptive activity and safety of AMG 162 were evaluated in 49 healthy postmenopausal women (Phase I study). The effect of increasing amounts of AMG 162 on bone mineral density (BMD) was studied in 411 postmenopausal women with low BMD (Phase II study). A single subcutaneous dose of AMG 162 (1 mg/kg or 60 mg) suppressed bone resorption for more than 6 month without critical side effects (Phase I study), and increased BMD within 1 month in postmenopausal women (Phase II study).

はじめに

骨芽細胞は，破骨細胞の分化に必要不可欠な 2 つのサイトカインである，macrophage colony-stimulating factor（以下 M-CSF と略す）と recep-

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tor activator of NF-κB ligand（以下 RANKL と略す）を発現する[1][2]。骨芽細胞は，構成的に M-CSF を発現するのに対し，RANKL を誘導的に発現する。すべての骨吸収を促進するホルモンやサイトカインは，骨芽細胞の RANKL の発現を誘導して骨吸収を促進する。RANKL は，tumor necrosis factor（以下 TNF と略す）ファミリーに属する膜結合型サイトカインである。さらに，骨芽細胞は，RANKL のデコイ受容体で，破骨細胞の形成を抑制する分泌性タンパク質 osteoprotegerin（以下 OPG と略す）も産生する。骨粗鬆症では，破骨細胞による骨吸収が，骨芽細胞による骨形成を凌駕するため骨量の低下が起こる。そのため，ビスホスホネートを始め多くの骨粗鬆症の治療薬は，骨吸収を抑制することを目的に開発されている。Amgen 社は，骨粗鬆症治療薬として OPG（AMGN-0007）とともに RANKL に対する完全なヒト抗体 AMG 162 の作製に成功した。米国で，AMG 162 を用いた臨床試験も進められている[3]~[5]。閉経後の女性を対象としたフェーズ I 試験では，AMG 162 単回皮下投注射によって，長期間（6 カ月以上）の骨吸収の抑制が持続することが報告された[3][4]。さらに，フェーズ II 試験では，骨量増加作用があることが示された[5]。

RANK/RANKL 系による破骨細胞制御機構

　1998 年，骨芽細胞が発現する破骨細胞分化因子がクローニングされ，TNF ファミリーに属する膜結合型タンパク質 RANKL であることが明らかとなった[6][7]。この発見により，骨吸収調節機構の一端が分子レベルで解明されるに至った[1][2]。すなわち，骨芽細胞は，破骨細胞の分化に必須な 2 つの因子 M-CSF と RANKL を発現することで，破骨細胞の形成を支持する（図 1）。破骨細胞前駆細胞は，単球・マクロファージ系細胞で，M-CSF 受容体と RANKL 受容体である receptor activator of NF-κB（以下 RANK と略す）を発現して

いる。破骨細胞前駆細胞は，細胞間接触機構で骨芽細胞が発現する RANKL を認識し，M-CSF の存在下で破骨細胞に分化する。また，成熟破骨細胞も RANK を発現しており，RANKL は破骨細胞の骨吸収活性を誘導する。活性型ビタミン D_3，副甲状腺ホルモン（以下 PTH と略す），プロスタグランディン E_2，インターロイキン 11 など，すべての骨吸収促進因子は，骨芽細胞における RANKL の発現を誘導する[1][2]。興味深いことに，骨芽細胞は，RANKL のデコイ受容体である分泌性タンパク質 OPG も産生し分泌する[1][2]。OPG は RANKL-RANK 相互作用を阻害し，骨吸収を強力に抑制する。RANKL 遺伝子と RANK 遺伝子の欠損マウスがそれぞれ作製された。これら欠損マウスは，ともに骨組織に破骨細胞が存在せず，重篤な大理石骨病を発症した[8][9]。これらの所見より，RANKL-RANK 相互作用は，破骨細胞の分化に必須であることが証明された。

RANKL の構造と抗 RANKL ヒト抗体 AMG 162

　RANKL は，316 個のアミノ酸からなる細胞膜貫通領域を持つ TNF ファミリーに属するサイトカインである[1][2]。Amgen 社は，ヒト RANKL に対する完全ヒトモノクローナ抗体 AMG 162 を作製した。AMG 162 の RANKL への結合は，Kd（解離定数）＝ 3×10^{-12}M と強く，RANKL-RANK 相互作用を強力に抑制する[4]。また，AMG 162 は TNF α，や TNF-related apoptosis-inducing ligand（以下 TRAIL と略す）などほかの TNF ファミリーメンバーとは結合しない。この抗体 AMG 162 を用いたフェーズ I 試験とフェーズ II 試験が米国で行われた。

AMG 162 フェーズ I 試験

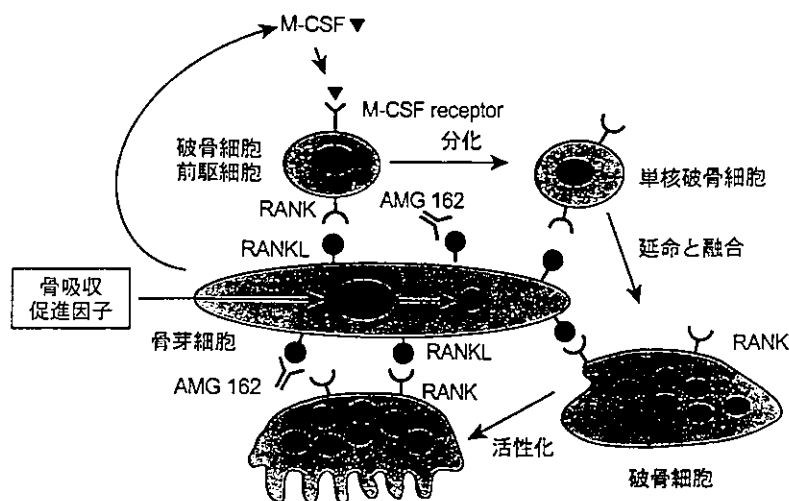　AMG 162 を用いたフェーズ I 試験では，49 人の閉経後の健常女性を対象に，その安全性と骨吸収抑制作用が検討された[3][4]。49 人を 7 グループ

図1 破骨細胞の形成と機能を誘導する RANKL と AMG 162 の作用

破骨細胞前駆細胞は，細胞間接触機構で骨芽細胞が発現する RANKL を認識し，M-CSF の存在下で破骨細胞に分化する。また，成熟破骨細胞も RANK を発現しており，RANKL は破骨細胞の骨吸収活性を誘導する。すべての骨吸収を促進する因子は，骨芽細胞の RANKL 発現を誘導する。RANKL に対する完全ヒトモノクローナ抗体 AMG 162 は，RANKL に結合し，RANKL-RANK 相互作用を抑制する。
RANK : receptor activator of NF-κB
RANKL : receptor activator of NF-κB ligand
M-CSF : macrophage colony-stimulating factor

（筆者ら作成）

に分け，AMG 162（0.01 mg/kg，0.03 mg/kg，0.1 mg/kg，0.3 mg/kg，1.0 mg/kg，3.0 mg/kg）あるいはプラセボを1回皮下投与し，骨代謝マーカーとして尿中NTX(N-telopeptide/creatinine)，血中NTX，血中 bone alkaline phosphatase(以下BALP と略す)を経時的に測定した。さらに，血清カルシウムとPTH を測定し，以下の結果を得た。

① AMG 162 投与により，尿中NTX 値は急激に(12 時間以内)そして容量依存的に低下した(図2)。高濃度投与群においては，この低下作用は6カ月以上も持続した。血中NTX も同様に低下した。

② BALPは緩やかに低下したが，6カ月以降に回復した（図3）。

③ 血清カルシウム値とPTH 値は，有意な変動を示さなかった。

④ 血中の AMG 162 濃度は，投与後徐々に減少

したが，9カ月後も 3.0 mg/kg 投与群において数十 ng/mL 見いだされた。AMG 162 は，消失されにくいことが明らかとなった（図4）。

これらの結果から，AMG 162 の骨吸収抑制効果はきわめて強く，6カ月以上骨吸収を抑制できることが判明した。AMG 162 の 3.0 mg/kg の単回投与でも，6カ月後の骨吸収抑制効果は，骨吸収抑制薬アレンドロネート（70 mg/ 週，10 mg/日）の投与よりも強かった（図5）。また，このフェーズ I 試験では，AMG 162 投与と関連のない腹痛1人と胆嚢炎1人を認めたほか，AMG 162 投与に起因する副作用は認められなかった。

AMG 162 フェーズ II 試験
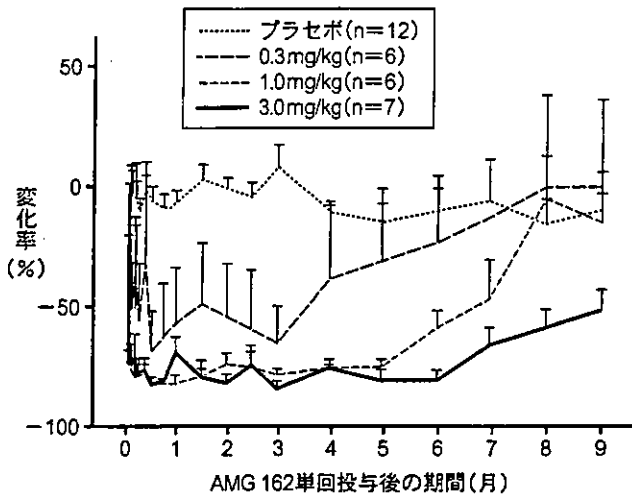
AMG 162 を用いたフェーズ II 試験が，腰椎の骨量が低い 411 人の閉経後の女性を対象に行われ

図2　AMG 162 単回投与後の尿中 NTX 値の経時変化
　　（フェーズⅠ試験）

　49 人の閉経後の健常女性を 7 群に分け，AMG 162
（0.01 mg/kg，0.03 mg/kg，0.1 mg/kg，0.3 mg/kg，1.0
mg/kg，3.0 mg/kg）あるいはプラセボを 1 回皮下投与し，
尿中 NTX（N-telopeptide/creatinine）を経時的に測定した。
データは変化率 % で表示した（平均値 ± SE）。プラセ
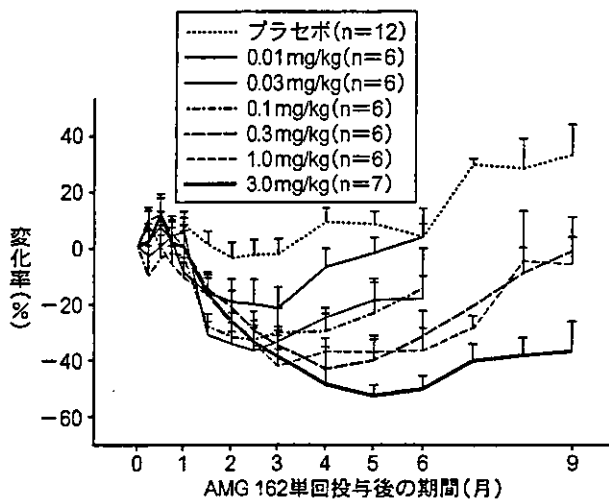ボ，AMG 162（0.3 mg/kg，1.0 mg/kg，3.0 mg/kg）投与群
の結果のみを記した。

（文献 3，4 より改変）

図3　AMG 162 単回投与後の血中 BALP（bone-
　　specific alkaline phosphatase）の経時変化
　　（フェーズⅠ試験）

　49 人の閉経後の健常女性に，AMG 162（0.01 mg/kg，
0.03 mg/kg，0.1 mg/kg，0.3 mg/kg，1.0 mg/kg，3.0 mg/kg）
あるいはプラセボを 1 回皮下投与し，血中 BALP を経時
的に測定した。低容量投与群（0.01 mg/kg，0.03 mg/kg，
0.1 mg/kg）は 6 カ月以降 BALP の測定はしていない。
データは変化率 % で表示した（平均値 ± SE）。

（文献 3，4 より改変）
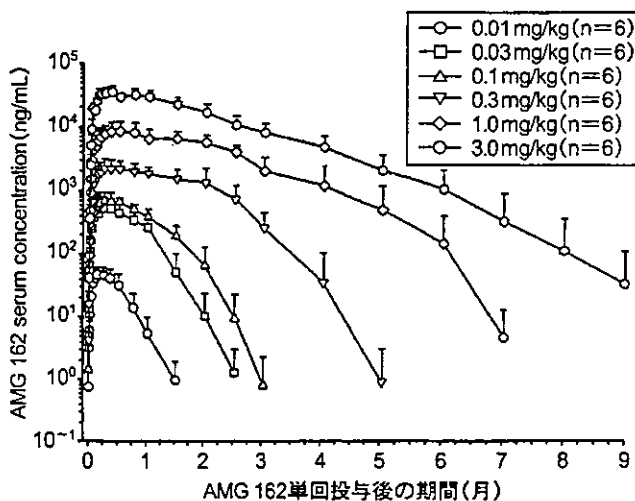
図4　AMG 162 単回投与における血中濃度の推移
　　（フェーズⅠ試験）

　データは 6 人の閉経後の健常女性の値を示す（平均
値 ± SE）。

（文献 3，4 より改変）
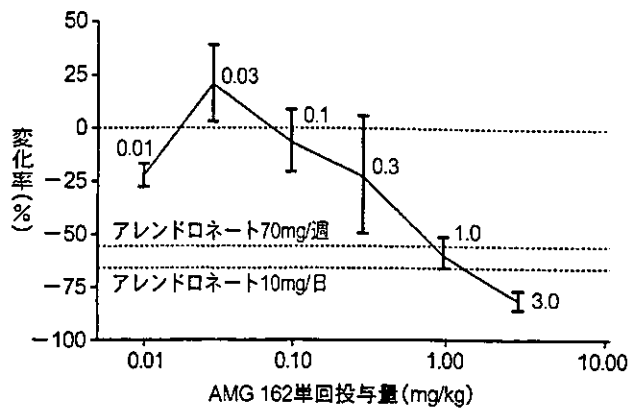
た[5]。AMG 162を3カ月ごとに6 mg，14 mg，30 mg，および6カ月ごとに14 mg，60 mg，100 mg，210 mgあるいはプラセボを皮下投与した。さらに1群の治験者には，アレンドロネート70 mgを週1回投与した。12カ月間骨量を経時的に測定し，次のことが明らかとなった。

① AMG 162を6カ月に1回投与した群では，最短で72時間で骨代謝マーカーの血清NTX値が減少し，アレンドロネート群よりも有意に低い状態を，14 mg群では2カ月間，それ以外の群では4カ月維持した（p < 0.0001）。

② 骨密度は，投与1カ月以内に増加した。AMG 162すべての投与群は投与量の増加に伴って増え，12カ月後において腰椎で4～7％の増加を示した（アレンドロネート投与群では5％の増加）。股関節部で2～4％増加した。

③ すべての群で最も多く見られた副作用は消化障害で，「AMG 162投与群」では5％，「アレンドロネート投与群」では20％，「プラセボ投与群」では4％だった。なお，AMG 162に対する抗体が2人の治験者で出現したものの，その後抗体は消滅し，治療効果に影響はなかったという。

今回の結果から，閉経後の女性の骨量を増加させるためには，60 mgのAMG 162を6カ月に1回投与するのが最も有効であるという[5]。

AMG 162の特徴

AMG 162に利点として，以下のことが挙げられる。

① マウス抗体やマウス-ヒトキメラ抗体と異なり，AMG 162は，完全なヒト抗体であるために血中に長く留まる。そのため，6カ月に一度の皮下注射で骨吸収抑制と骨量の十分な増加が認められる。

② OPGは，RANKL以外にTRAILと結合することが報告されているが，AMG 162は，RANKL以外の他のTNFファミリーメンバーとは結合しない。

③ AMG 162はヒト抗体であるために，AMG 162に対する抗体ができにくい。また，抗AMG 162抗体ができても，OPG-RANKLの相互作用を阻害することはない。

おわりに

以上のように，完全にヒト型のRANKL抗体AMG 162は，骨粗鬆症に対して有効な治療薬となりえることが報告された。さらに，癌関連の骨病変（骨転移のある乳癌患者）へのAMG 162の投与も行われており，こちらも有効であると報告された[10]。AMG 162は，RANKLを直接ブロックす

－207－

る治療薬で，強い骨量増加作用を有するとともに副作用がないことから，骨粗鬆症を始め他の骨疾患の治療薬として認可される可能性が高いと思われる。RANKLの発見から6年目で，そのRANKLを標的とした効果的な治療薬が出現した。驚くべきスピードで研究と開発が進んでいることを示すものである。

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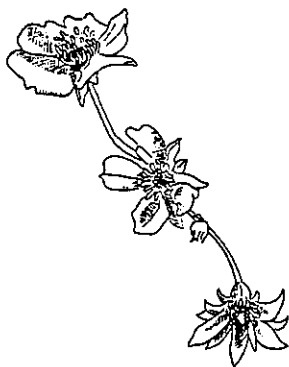
第1回　破骨細胞の分化と機能の調節機構

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キーワード　RANKL, TRAF, NFATc1, OSCAR, DAP12

Summary　骨組織は骨吸収と骨形成をくり返し，常に新しい組織に置きかえられる．骨吸収を担う破骨細胞は，骨芽細胞が発現するM-CSFおよびRANKLにより，マクロファージから分化する．近年，破骨細胞前駆細胞膜上に発現するOSCARやTREM-2などの免疫受容体と，骨芽細胞あるいは破骨細胞前駆細胞の細胞膜上に発現するリガンドとの相互作用も破骨細胞の分化に重要であることが示された．これらの免疫受容体は，ITAMモチーフを有するアダプター分子FcRγやDAP12と共役してはたらき，その下流で破骨細胞分化のキーファクターであるNFATc1を活性化する．これらアダプター分子のダブルノックアウトマウスは，破骨細胞の形成阻害による重篤な大理石骨病を呈する．

略語一覧

M-CSF : macrophage-colony stimulating factor, RANKL : receptor activator of NF-κB ligand, OSCAR : osteoclast-associated receptor, TREM-2 : triggering receptor expressed by myeloid cells-2, ITAM : immunoreceptor tyrosine based activation motif, DAP12 : DNAX-activation protein 12, FcRγ : Fc receptor common γ subunit, NFATc1 : nuclear factor of activated T cell c1, TRAF : TNF receptor-associated factor, RANK : receptor activator of NF-κB, JNK : c-jun N-terminal kinase, p38MAPK : p38 mitogen-activated protein kinase, ERK : extracellular signal regulated kinase, VDR : vitamin D receptor, PTH : parathyroid hormone, PGE₂ : prostaglandin E₂, PKA : protein kinase A, IL-6 : interleukin-6, IL-11 : interleukin-11, IL-1 : interleukin-1, LPS : lipopolysaccharide, PKC : protein kinase C, TLR-4 : Toll-like receptor4, PLCγ : phospholipase Cγ

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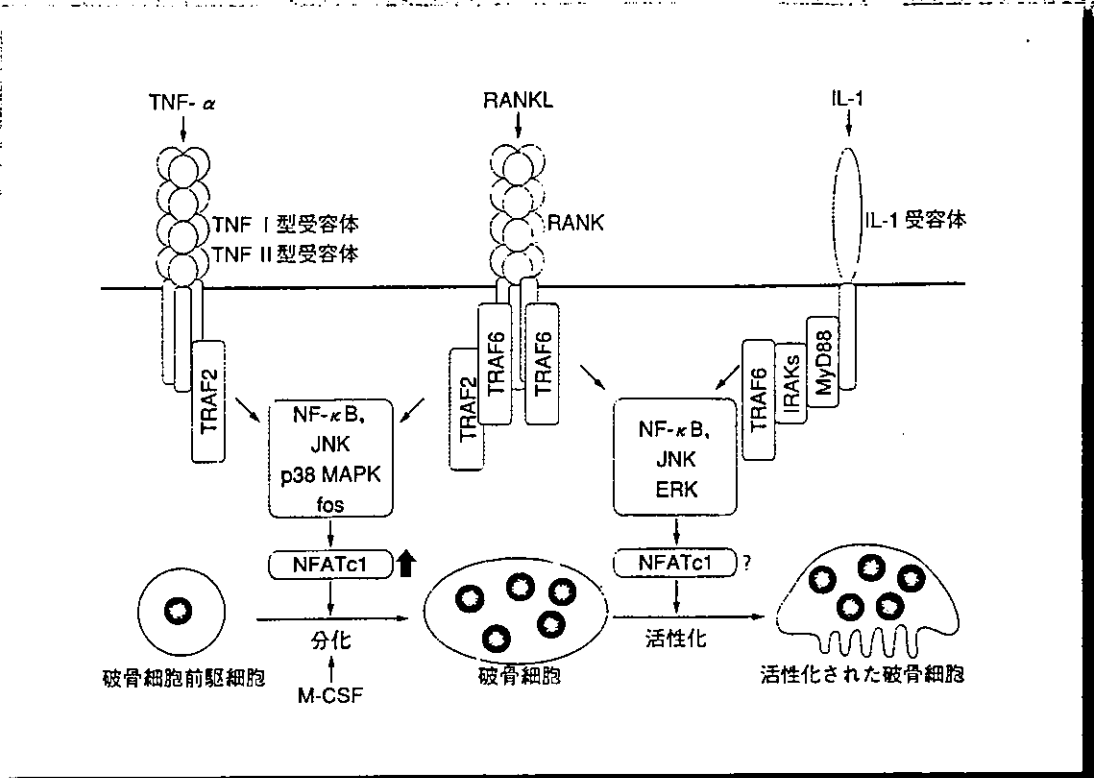
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図1　破骨細胞の分化と機能発現を誘導するRANKL，TNF-αおよびIL-1のシグナル系

解説

骨芽細胞が発現するRANKLとM-CSFは破骨細胞の分化に必須なサイトカインである．骨芽細胞が提示するRANKLは，破骨細胞前駆細胞あるいは成熟破骨細胞が発現する受容体RANKを介して，破骨細胞の分化と活性化を誘導する．RANKシグナルは，シグナル伝達因子TRAFを介してNF-κB，JNK，p38MAPK，ERK，fosなどを活性化する．TNF-αは破骨細胞の分化のみを誘導し，IL-1は破骨細胞の機能のみを促進する．このことから，破骨細胞の分化にはTRAF2が，機能にはTRAF6がはたらくように思われたが，破骨細胞分化におけるTRAF6の重要性も指摘されている．破骨細胞の分化誘導には，転写因子NFATc1の活性化が必須である．一方，NFATc1が破骨細胞の機能発現に関与するか否かは不明である．
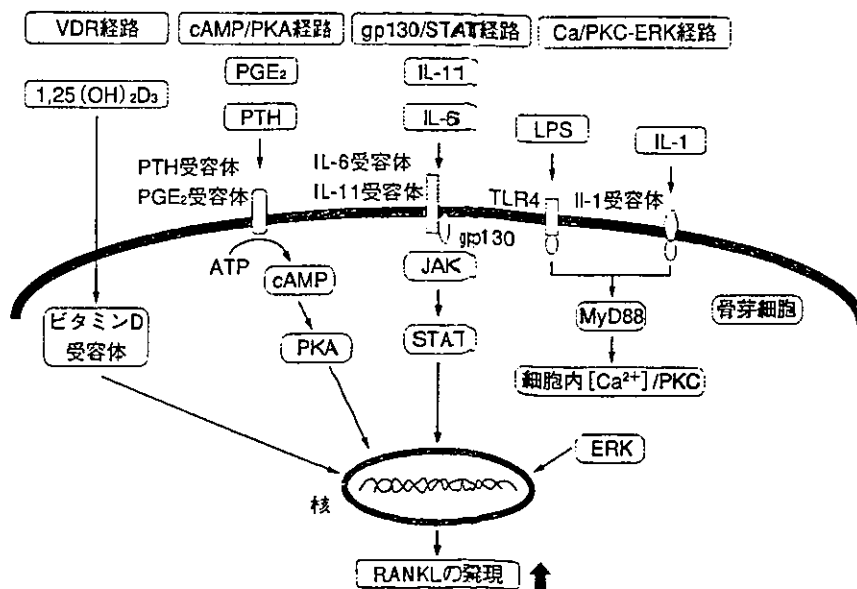
図2　RANKL発現を誘導する4つのシグナル系

解説

骨芽細胞におけるRANKL発現を誘導する経路は，①活性型ビタミンD₃〔1.25(OH)₂D₃〕のシグナルを伝達するvitamin D receptor(VDR)経路，②副甲状腺ホルモン(PTH)やPGE₂のシグナルを伝達するcAMP/PKA経路，③IL-6やIL-11のシグナルを伝達するgp130/STAT3経路，④IL-1やLPSのシグナルを伝達するCa/PKC-ERK経路が存在する．LPS受容体(TLR4)とIL-1受容体の下流にはMyD88が存在し，IL-1やLPSによるRANKL発現誘導はMyD88を介することが示されている．
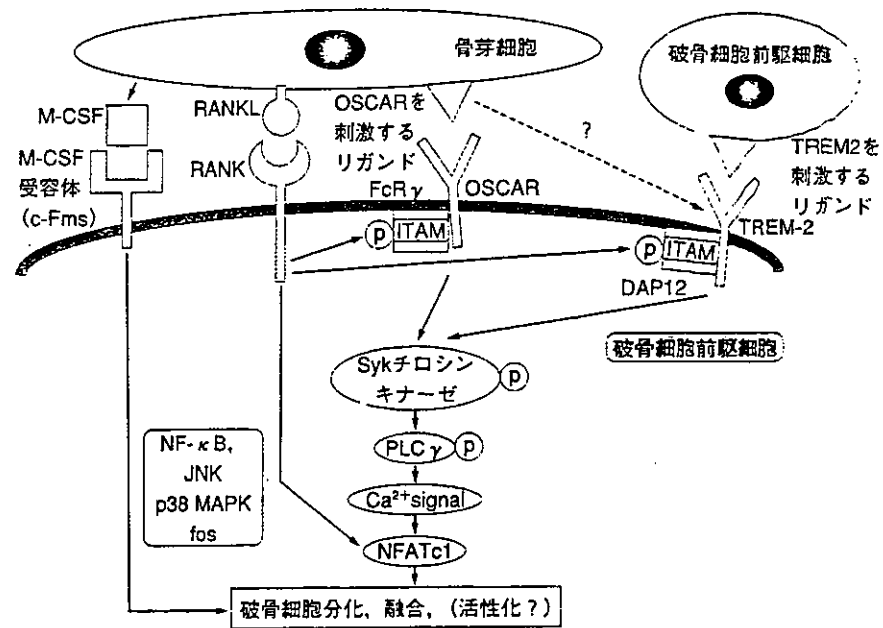
4(4)

図3　破骨細胞形成におけるITAMを介するシグナル系

解説

近年，破骨細胞前駆細胞の細胞膜上に発現するOSCARやTREM-2など
の免疫受容体と，骨芽細胞あるいは破骨細胞前駆細胞の細胞膜上に発現
するリガンドとの相互作用も破骨細胞の分化に重要であることが示され
た．RANKL刺激はFcRγやDAP12のITAMモチーフのチロシンリン
酸化を誘導する．そのチロシンリン酸化は非受容体型チロシンキナーゼ
のSykをリクルートする．SykはPLCγを活性化し，細胞内Caシグナ
ルを誘導する．この細胞内Caシグナルは，転写因子NFATc1を活性化
し，RANKLとM-CSFにより誘導される破骨細胞の分化を促進する．
FcRγとDAP12のダブルノックアウトマウスは，破骨細胞の形成が著
しく抑制された大理石骨病を呈する．骨芽細胞がTREM-2を刺激でき
るか否かは不明である．

Suppression of Osteoprotegerin Expression by Prostaglandin E₂ Is Crucially Involved in Lipopolysaccharide-Induced Osteoclast Formation[1]

Koji Suda,*[†] Nobuyuki Udagawa,[‡] Nobuaki Sato,[‡] Masamichi Takami,[†] Kanami Itoh,[†] Je-Tae Woo,[¶] Naoyuki Takahashi,[2][§] and Kazuo Nagai[¶]

LPS is a potent stimulator of bone resorption in inflammatory diseases. The mechanism by which LPS induces osteoclastogenesis was studied in cocultures of mouse osteoblasts and bone marrow cells. LPS stimulated osteoclast formation and PGE₂ production in cocultures of mouse osteoblasts and bone marrow cells, and the stimulation was completely inhibited by NS398, a cyclooxygenase-2 inhibitor. Osteoblasts, but not bone marrow cells, produced PGE₂ in response to LPS. LPS-induced osteoclast formation was also inhibited by osteoprotegerin (OPG), a decoy receptor of receptor activator of NF-κB ligand (RANKL), but not by anti-mouse TNFR1 Ab or IL-1 receptor antagonist. LPS induced both stimulation of RANKL mRNA expression and inhibition of OPG mRNA expression in osteoblasts. NS398 blocked LPS-induced down-regulation of OPG mRNA expression, but not LPS-induced up-regulation of RANKL mRNA expression, suggesting that down-regulation of OPG expression by PGE₂ is involved in LPS-induced osteoclast formation in the cocultures. NS398 failed to inhibit LPS-induced osteoclastogenesis in cocultures containing OPG knockout mouse-derived osteoblasts. IL-1 also stimulated PGE₂ production in osteoblasts and osteoclast formation in the cocultures, and the stimulation was inhibited by NS398. As seen with LPS, NS398 failed to inhibit IL-1-induced osteoclast formation in cocultures with OPG-deficient osteoblasts. These results suggest that IL-1 as well as LPS stimulates osteoclastogenesis through two parallel events: direct enhancement of RANKL expression and suppression of OPG expression, which is mediated by PGE₂ production. *The Journal of Immunology,* 2004, 172: 2504–2510.

O steoclasts are bone-resorbing multinucleated cells that originate from hemopoietic progenitors of the monocyte/macrophage lineage (1–4). Osteoblasts or bone marrow stromal cells are involved in osteoclastogenesis through a mechanism involving cell-to-cell contact with osteoclast progenitors (4, 5). Studies of M-CSF-deficient *op/op* mice have shown that M-CSF produced by osteoblasts is an essential factor for osteoclastogenesis (6, 7). Receptor activator of NF-κB ligand (RANKL)[3] (3) was also identified as another factor essential for osteoclastogenesis (8–11). RANKL is a member of the TNF-ligand family that is expressed by osteoblasts/stromal cells as a membrane-associated factor. Osteoclast precursors express RANK, a receptor of RANKL; recognize RANKL through cell-cell interaction; and differentiate into osteoclasts in the presence of M-CSF (12, 13). Os-

teoblasts/Stromal cells also produce a soluble decoy receptor for RANKL, osteoprotegerin (OPG), which inhibits osteoclast formation in vivo and in vitro by interrupting the interaction between RANKL and RANK (14, 15).

In mouse cell cocultures, osteoclasts are formed in response to bone-resorbing factors such as 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), parathyroid hormone (PTH), PGE₂, and IL-11 (4). Almost all of the bone-resorbing factors stimulate expression of RANKL in osteoblasts/stromal cells (4). Three independent signals have been proposed to induce RANKL expression in osteoblasts/stromal cells: vitamin D receptor-mediated signals induced by 1,25(OH)₂D₃, cAMP/protein kinase A (PKA)-mediated signals induced by PTH or PGE₂, and gp130-mediated signals induced by IL-11 (4). Among these signals, vitamin D receptor- and cAMP/PKA-mediated signals suppress OPG expression in osteoblasts/stromal cells. Recently, we reported that compounds that elevate intracellular calcium, such as ionomycin, A23187, cyclopiazonic acid, and thapsigargin, stimulated osteoclast formation in mouse cocultures (16). Treatment of primary osteoblasts with those compounds stimulated the expression of RANKL. Thus, the signal mediated by calcium and protein kinase C (PKC) is proposed to be another (fourth) signal that induces RANKL expression in osteoblasts/stromal cells.

Severe bone loss due to excessive bone resorption is observed in inflammatory diseases such as periodontitis and osteomyelitis and some types of arthritides (17). LPS, a major constituent of Gram-negative bacteria, is proposed to be a potent stimulator of bone loss in these inflammatory diseases (17–22). Recently, Toll-like receptor 4 (TLR4) was identified as the signal-transducing receptor for LPS (23, 24). The cytoplasmic signaling cascade of TLR4 is similar to that of IL-1Rs. Both TLR4 and IL-1Rs use common signaling molecules such as myeloid differentiation factor 88 and TNFR-associated factor 6 (25–29). Macrophages, lymphocytes,

and osteoblasts/stromal cells express TLR4, and produce PGE_2 and proinflammatory cytokines such as TNF-α and IL-1 in response to LPS (30, 31). These inflammatory factors also stimulate osteoclastogenesis directly or indirectly (17–22, 32).

LPS stimulates PGE_2 production in the target cells through the induction of mitogen-inducible cyclooxygenase 2 (COX2) expression (33). NS398, a nonsteroidal anti-inflammatory agent, specifically inhibits COX2 without affecting COX1 activity (34). Therefore, NS398 has been used as a tool to explore the role of PGE_2 in pathological processes involving COX2 activity. PGE_2 exerts its biological actions through binding to four specific membrane receptors (EP1, EP2, EP3, and EP4) (35). Sakuma et al. (20, 21) reported that induction of osteoclast formation by LPS, TNF-α, and IL-1 was barely observed in cell cultures prepared from EP4 knockout (EP4$^{-/-}$) mice, and that urinary excretion of deoxypyridinoline, a sensitive marker for bone resorption, was not increased in EP4$^{-/-}$ mice injected with LPS. These results suggest that PGE_2 is a key factor in the enhancement of osteoclastogenesis by LPS in vivo and in vitro. However, it is still not known how PGE_2 is involved in the induction of osteoclastogenesis by LPS.

In the present study, we examined the mechanism of the induction of osteoclast formation by LPS in cocultures of mouse osteoblasts and bone marrow cells. We showed that LPS promoted osteoclastogenesis through two parallel events: one was direct enhancement of RANKL expression, and the other was suppression of OPG production mediated by PGE_2 in osteoblasts. In addition, IL-1 stimulated osteoclast formation in the cocultures in a manner similar to LPS.

Materials and Methods

Reagents and mice

LPS (*Escherichia coli* O26:B6) and PGE_2 were purchased from Sigma-Aldrich (St. Louis, MO). NS398 was from Calbiochem (San Diego, CA). Human rOPG and mouse rIL-1β were obtained from PeproTech (London, U.K.). Mouse rTNF-α and mouse rIL-1 receptor antagonist (rIL-1ra) were obtained from R&D Systems (Minneapolis, MN). Anti-mouse TNFR1 Ab was obtained from Genzyme Diagnostics (Cambridge, MA). Six- to 9-wk-old male and newborn ddY mice were obtained from Sankyo Laboratory Animal Center (Tokyo, Japan). C57BL/6 (B6) mice and OPG-deficient (OPG$^{-/-}$) mice (C57BL/6 (B6)) were obtained from Clear Japan Clea (Tokyo, Japan). This study was reviewed and approved by the Showa University Animal Care and Use Committee.

Cell preparation and osteoclast formation assay

Primary osteoblasts were obtained from calvariae of newborn ddY mice, C57BL/6 (B6) mice, and OPG$^{-/-}$ mice by the conventional method using collagenase (36). Bone marrow cells were collected from femora and tibiae of 6- to 9-wk-old male mice. Primary osteoblasts (1×10^4 cells) and bone marrow cells (2×10^5 cells) were cocultured for 5 days in α-MEM containing 10% FCS (CSL, Victoria, Australia) in 96-well tissue culture plates (Corning, Corning, NY) (0.2 ml/well). Cocultures were incubated in the presence of LPS (0.001–10 μg/ml), PGE_2 (1 μM), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) for the final 3 days. Some cocultures were pretreated with NS398 (1 μM), OPG (100 ng/ml), IL-1ra (10 μg/ml), and TNFR1 Ab (10 μg/ml) for 1 h before adding LPS, PGE_2, IL-1β, or TNF-α. Then cocultures were fixed and stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts). TRAP-positive cells containing >3 nuclei were counted as osteoclasts. The results obtained from a typical experiment of three independent experiments are expressed as the mean ± SD of four cultures.

Measurement of PGE_2 production

Primary osteoblasts (3×10^4 cells) and bone marrow cells (6×10^5 cells) were cultured separately or in combination with or without LPS (1 μg/ml) or IL-1β (10 ng/ml) in α-MEM containing 10% FCS in 48-well culture plates (Corning). After the cultures were incubated for 6 h, the concentration of PGE_2 in the culture medium was determined using an enzyme immunoassay (EIA; Cayman Chemicals, Ann Arbor, MI). The Ab showed the following cross-reactivity determined by comparing the bond/free ra-

tios with several eicosanoids: PGE_2, 100%; PGE_2 ethanolamide, 100%; PGE_3, 43%; PGE_1, 18.7%; 6-keto $PGF_1\alpha$, 1%; and 8-*iso* $PGF_2\alpha$, 0.25%.

Northern blot analysis

Primary osteoblasts (1×10^6 cells) were seeded in cell culture dishes (60 mm in diameter; Corning) and cultured in α-MEM containing 10% FCS for 3 days. After incubation in α-MEM containing 0.1% FCS for 3 h, the cells were incubated with LPS (1 μg/ml) or IL-1β (10 ng/ml). In some experiments, osteoblasts were cocultured with bone marrow cells (2×10^7 cells) in the presence of LPS (1 μg/ml) for 3 or 48 h. Some cultures were also treated with NS398 (1 μM) for 1 h before the addition of LPS. Total RNA was isolated from cultures using TRIzol (Life Technologies, Grand Island, NY). Northern blot analysis was performed using denaturing formaldehyde/agarose gels, as described (16). Double-stranded cDNA fragments encoding mouse RANKL OPG and COX2 were kindly provided by H. Yasuda (Snow Brand Milk Products, Tochigi, Japan). cDNA probes (RANKL, OPG, COX2, and β-tubulin) labeled with ^{32}P were synthesized using a cDNA labeling kit (Takara, Tokyo, Japan). The RANKL, OPG, COX2, and β-tubulin probes were hybridized with membranes to which total RNA isolated from osteoblasts had been transferred. The membranes were exposed to Kodak BioMax MS film (Rochester, NY) for 3–48 h. Signals of RANKL, OPG, COX2, and β-tubulin mRNA were quantified using a radioactive image analyzer (BAS2000; Fuji Photo Film, Tokyo, Japan). Signals of RANKL, OPG, and COX2 mRNAs were normalized with the respective β-tubulin mRNA expression levels to calculate the relative intensity.

Results

PGE_2 is required for osteoclast formation induced by LPS

LPS stimulated TRAP-positive osteoclast formation in cocultures of primary osteoblasts and bone marrow cells in a dose-dependent manner (Fig. 1A). The maximal number of osteoclasts was observed at 1 μg/ml of LPS. We then examined whether PGE_2 is involved in the induction of osteoclast formation by LPS. LPS (1 μg/ml) as well as PGE_2 (1 μM) induced TRAP-positive osteoclast formation in the cocultures (Fig. 1, *B* and *C*). NS398 (1 μM), a specific inhibitor of COX2, suppressed the induction of osteoclast formation by LPS, but not by PGE_2 in the cocultures (Fig. 1, *B* and *C*). Both LPS- and PGE_2-induced osteoclast formation in the cocultures was strongly inhibited by simultaneous addition of OPG (100 ng/ml) (Fig. 1, *B* and *C*). These results suggest that both PGE_2 production and RANKL-RANK interaction are required for LPS-induced osteoclast formation in the cocultures. We next examined whether LPS induces osteoclastogenesis through IL-1 and TNF-α. Recombinant IL-1β (10 ng/ml) and TNF-α (10 ng/ml) induced osteoclastogenesis in the cocultures. IL-1ra (10 μg/ml) and anti-mouse TNFR1 Ab (10 μg/ml) strongly inhibited the osteoclast formation induced by IL-1β and TNF-α, respectively. However, neither IL-1ra nor TNFR1 Ab affected osteoclastogenesis induced by LPS (Fig. 1D). These results suggest that PGE_2 is a critical factor in LPS-induced osteoclastogenesis.

Osteoblasts mainly produce PGE_2 in response to LPS

We then measured the PGE_2 concentration in the conditioned medium of cocultures incubated with or without LPS (1 μg/ml) for 6 h (Fig. 2A). LPS significantly increased the PGE_2 concentration in the culture medium (Fig. 2A). The addition of NS398 (1 μM) to the cocultures completely blocked the induction of PGE_2 production by LPS in the cocultures (Fig. 2A). To determine the type of cells that respond to LPS in the cocultures, osteoblasts and bone marrow cells were cultured separately in the presence or absence of LPS for 6 h (Fig. 2B). LPS stimulated PGE_2 production in the cultures of osteoblasts, but not bone marrow cells. LPS-induced PGE_2 production was strongly inhibited by the addition of NS398 (Fig. 2B). Northern blot analysis showed that treatment of osteoblasts with LPS for 3 h stimulated the expression of COX2 mRNA (Fig. 2C). These results suggest that osteoblasts in the cocultures

FIGURE 1. Effects of NS398, OPG, IL-1ra, and
TNFR1 Ab on osteoclast formation in cocultures
treated with LPS. A, Mouse primary osteoblasts and
bone marrow cells were cocultured for 5 days. LPS
(0.001–10 μg/ml) was added to the cocultures for
the final 3 days. TRAP-positive multinucleated cells
containing more than three nuclei were counted as
osteoclasts. Values are expressed as the means ± SD
of quadruplicate cultures. B, LPS (1 μg/ml) or PGE2
(1 μM) together with or without NS398 (1 μM) or
OPG (100 ng/ml) was added to the cocultures for the
final 3 days. The cells were then fixed and stained
for TRAP. Arrowheads indicate TRAP-positive
multinucleated cells. Bar, 200 μm. C, TRAP-positive
multinucleated cells containing more than three nuclei
were counted as osteoclasts. Values are expressed as
the means ± SD of quadruplicate cultures. D, LPS (1
μg/ml), IL-1β (10 ng/ml), or TNF-α (10 ng/ml) to-
gether with or without IL-1ra (10 μg/ml) and TNFR1
Ab (10 μg/ml) was added to the cocultures for the final
3 days. TRAP-positive multinucleated cells containing
more than three nuclei were counted as osteoclasts.
Values are expressed as the means ± SD of quadru-
plicate cultures.
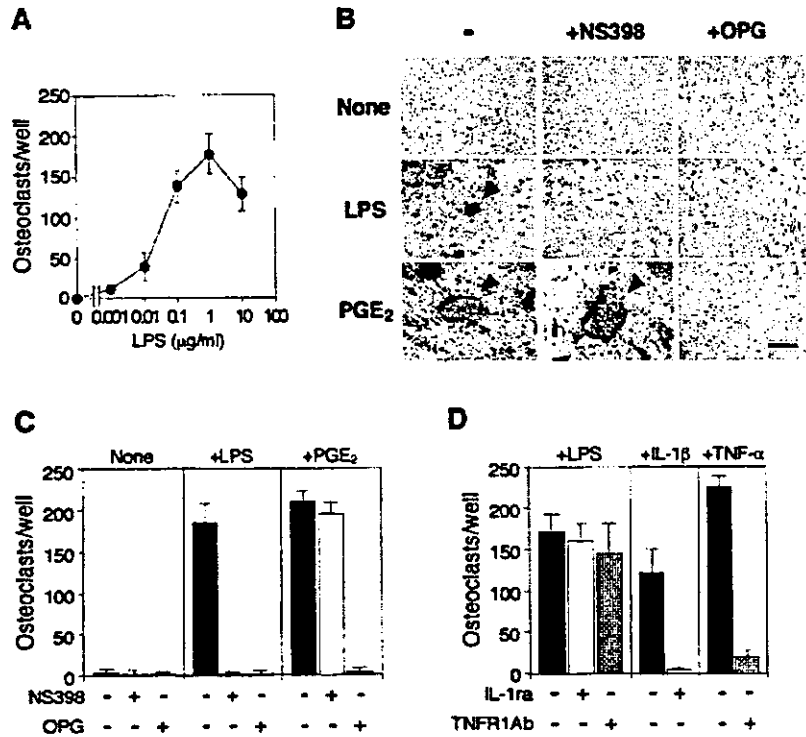
produce PGE2 in response to LPS via up-regulation of COX2
mRNA expression.

LPS regulates RANKL and OPG gene expression in osteoblasts

We next analyzed the effects of LPS on RANKL and OPG mRNA
expression levels in primary osteoblasts by Northern blot analysis
(Fig. 3). Treatment of the osteoblasts with LPS increased RANKL
mRNA expression with two peaks at 3 and 48 h. The expression of
RANKL mRNA after treatment with LPS was increased within
1 h, and was still higher than that of the control cultures even after
72 h (Fig. 3A). The expression of OPG mRNA in osteoblasts was
also enhanced by the treatment with LPS for 3 h (Fig. 3A). How-
ever, the expression of OPG mRNA in osteoblasts treated with
LPS for 48 or 72 h was decreased to a level lower than that of the
control culture (Fig. 3A). NS398 (1 μM) had no effect on the level
of RANKL mRNA induced by LPS at 3 h (Fig. 3B). The LPS-
induced up-regulation of RANKL mRNA expression at 48 h was
slightly inhibited by the COX2 inhibitor, but the level of the
mRNA was much higher than that in the control cultures. In con-
trast, the LPS-induced down-regulation of OPG mRNA expression
in osteoblasts at 48 h after treatment with LPS was completely
blocked by the addition of NS398, although the OPG mRNA ex-
pression at 3 h was not affected by the COX2 inhibitor (Fig. 3B).
The expression levels of RANKL and OPG mRNAs in bone mar-
row cells were lower than those in primary osteoblasts, and were
unchanged even after treatment with NS398 for 3 or 48 h (data not
shown). These results suggest that PGE2 produced by osteoblasts
plays an important role in the down-regulation of OPG expression,
but not the up-regulation of RANKL expression in osteoblasts
treated with LPS.

*Suppression of OPG expression is involved in induction of
osteoclast formation by LPS*

We next examined how PGE2 production is involved in LPS-in-
duced osteoclast formation using osteoblasts from OPG-deficient
(OPG−/−) mice. Primary osteoblasts prepared from OPG−/− mice

were cocultured with bone marrow cells from wild-type mice in
the presence or absence of NS398, OPG, and/or LPS (Fig. 4). In
agreement with previously reported findings (37), TRAP-positive
osteoclasts were formed in cocultures containing OPG−/− osteo-
blasts even in the absence of any stimulus (Fig. 4). The number of
osteoclasts was further increased in the LPS-treated cocultures
containing OPG−/− osteoblasts. NS398 strongly suppressed the
spontaneous osteoclast formation in the control cocultures con-
taining OPG−/− osteoblasts. This suggests that endogenous
production of PGE2 plays an important role in the osteoclast
formation in cocultures containing OPG−/− osteoblasts. NS398
slightly, but not completely, inhibited LPS-induced osteoclast
formation in cocultures containing OPG−/− osteoblasts (Fig. 4).
OPG completely suppressed osteoclast formation in the cocul-
tures treated or not treated with LPS (Fig. 4). These results
suggest that the down-regulation of OPG expression by PGE2 is
crucially involved in the osteoclast formation induced by LPS
in the cocultures.

IL-1 stimulates osteoclast formation in a manner similar to LPS

Because the signaling pathway of IL-1Rs is quite similar to that of
TLR4 (26–30), we finally examined whether IL-1 stimulates os-
teoclastogenesis in the cocultures in a manner similar to LPS.
IL-1β (10 ng/ml) induced osteoclast formation in the wild-type
cocultures, and the induction was inhibited by NS398 (1 μM) and
OPG (100 ng/ml) (Fig. 5A). IL-1β (10 ng/ml) also stimulated
PGE2 production in osteoblasts, but not in bone marrow cells after
treatment for 6 h (Fig. 5B). Northern blot analysis showed that
IL-1β up-regulated COX2 mRNA expression in osteoblasts at 3 h
(Fig. 5C). IL-1β also stimulated RANKL mRNA expression at 3 h
(data not shown). IL-1β stimulated osteoclast formation in the co-
cultures of OPG−/− osteoblasts and wild-type bone marrow cells
(the control: 53 ± 11, the mean ± SD of four cultures) (Fig. 5D).
NS398 (1 μM) did not completely suppress LPS-induced oste-
oclast formation in the cocultures with OPG−/− osteoblasts, but

A

B

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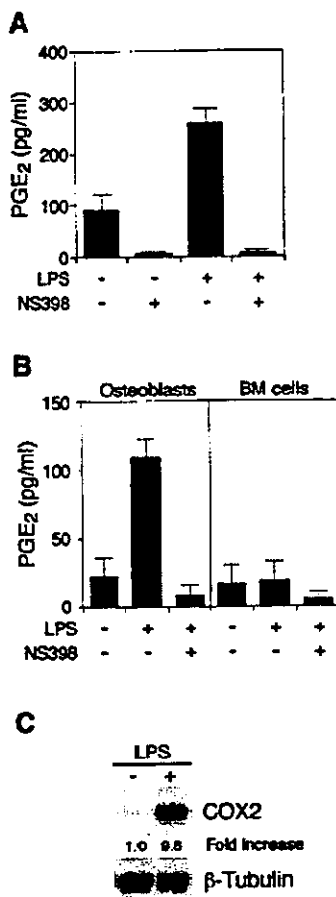
FIGURE 2. LP$ induces PGE$_2$ production and COX2 expression in osteoblasts. A, Primary osteoblasts and bone marrow (BM) cells were cocultured with LPS (1 µg/ml) in the presence or absence of NS398 (1 µM). After the cultures were incubated for 6 h, the concentration of PGE$_2$ in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. B, Primary osteoblasts and bone marrow cells were cultured separately with LPS (1 µg/ml) in the presence or absence of NS398 (1 µM) for 6 h. The PGE$_2$ concentration in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. C, Primary osteoblasts were treated with LPS (1 µg/ml) for 3 h. Total RNA was isolated from the osteoblasts, and COX2 and β-tubulin mRNA expression was analyzed by Northern blotting. Figures below the signals represent the intensity of the COX2 mRNA signals relative to the β-tubulin mRNA signals.

OPG did (Fig. 5D). These results suggest that IL-1 and LPS stimulate osteoclast formation in the same manner in the cocultures.

Discussion

In vivo and in vitro experiments have shown that PGE$_2$ is crucially involved in the induction of osteoclastic bone resorption by IL-1, TNF-α, and LPS (20). EP4 subtype-mediated signaling has been shown to be particularly important for the induction of bone resorption by such inflammation-related factors as well as PGE$_2$ (20). The present study showed that LPS stimulated COX2 expression and PGE$_2$ production in osteoblasts, and NS398, a specific inhibitor of COX2, strongly blocked the LPS-induced osteoclast formation in cocultures containing wild-type osteoblasts (Figs. 1 and 2). These results suggest that PGE$_2$ is somehow involved in LPS-induced osteoclast formation in the cocultures through PGE$_2$ receptors of EP4 subtype.

It was reported that LPS stimulated the expression of RANKL mRNA in osteoblasts obtained from EP4$^{-/-}$ mice, and that COX

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B
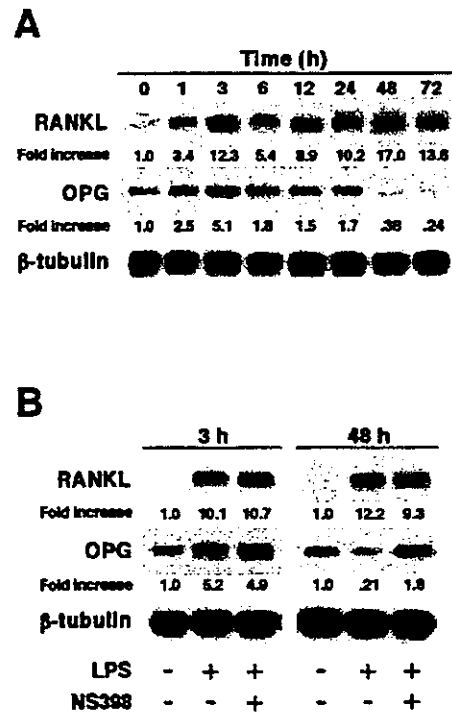
FIGURE 3. LPS regulates the expression of RANKL and OPG mRNAs in osteoblasts. A, Primary osteoblasts were treated with LPS (1 µg/ml) for 0–72 h. Total RNA was isolated from the osteoblasts, and the expression of RANKL, OPG, and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of the RANKL and OPG mRNA signals relative to the β-tubulin mRNA signals. B, Primary osteoblasts were treated with LPS (1 µg/ml) for 3 or 48 h in the presence of bone marrow cells. NS398 (1 µM) was also added to some cultures. After incubation for the indicated periods, bone marrow cells were removed by pipetting. Total RNA was isolated from osteoblasts, and the expression of RANKL, OPG, and β-tubulin mRNAs was analyzed by Northern blotting. Figures below the signals represent the intensity of the RANKL and OPG mRNA signals relative to the β-tubulin mRNA signals.

inhibitors did not block this stimulation (21). In agreement with this finding, NS398 failed to inhibit the induction of RANKL expression by LPS in osteoblasts (Fig. 3). These results suggest that LPS induced RANKL expression in a manner that was independent of PGE$_2$ production in osteoblasts. In contrast, the treatment of osteoblasts in the cocultures with LPS together with NS398 blocked the down-regulation of OPG mRNA expression at 48 h (Fig. 3). This suggests that suppression of OPG by PGE$_2$ is an important event in osteoclast formation in the cocultures treated with LPS. This notion was further supported by the finding that LPS stimulated osteoclast formation even in the presence of NS398 in cocultures containing osteoblasts derived from OPG$^{-/-}$ mice (Fig. 4). Thus, PGE$_2$ appears to play an important role as a suppressor of OPG expression rather than an activator of RANKL expression in LPS-induced osteoclast formation (Fig. 6). Recently, Fu et al. (38) reported that the activation of CREB by PTH is required for PTH-induced down-regulation of OPG expression. This suggests that the cAMP-PKA signals play a role in PGE$_2$-induced suppression of OPG mRNA expression. Further study will elucidate the detail mechanism of the down-regulation of OPG expression by PGE$_2$.

PGE$_2$ has been shown to induce RANKL mRNA expression in osteoblasts (10). Suzawa et al. (39) reported that PGE$_2$-induced RANKL expression is mediated through the cAMP signaling pathway. In our experiments, NS398 failed to inhibit RANKL expression in osteoblasts treated with LPS for as long as 48 h (Fig. 3).
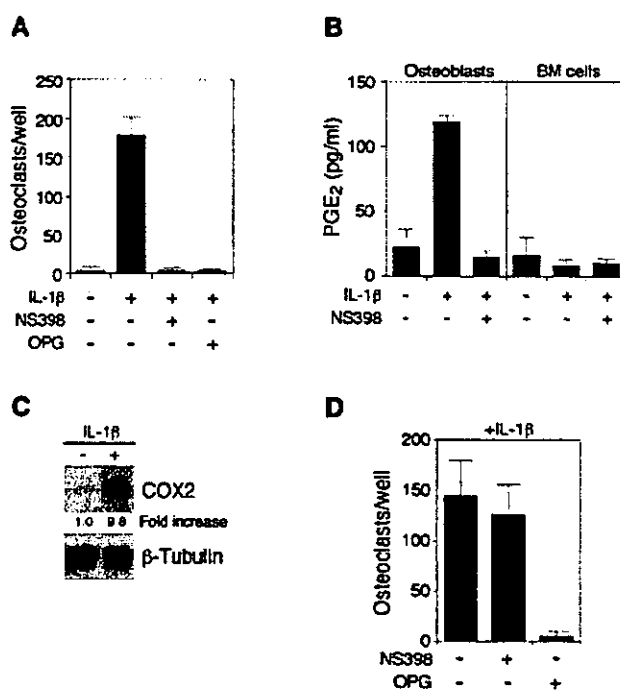
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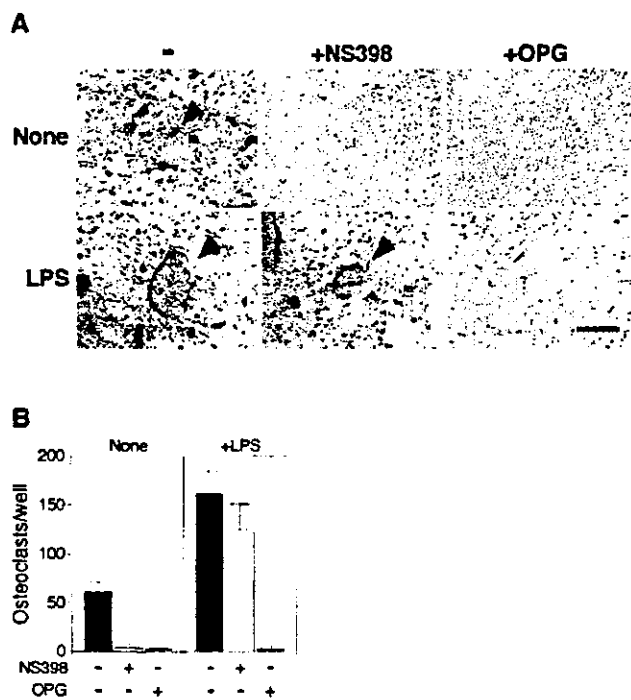
FIGURE 4. Effects of NS398 on LPS-induced osteoclast formation in cocultures containing OPG$^{-/-}$ mouse-derived osteoblasts. *A*, Primary osteoblasts prepared from OPG$^{-/-}$ mice were cocultured with wild-type bone marrow cells for 5 days. LPS (1 μg/ml) was added to the cocultures with or without NS398 (1 μM) or OPG (100 ng/ml) for the final 3 days. The cells were then fixed and stained for TRAP. Arrowheads indicate the TRAP-positive osteoclasts. Bar, 200 μm. *B*, TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

A

B

C

D

FIGURE 5. IL-1 induces osteoclast formation in the cocultures in a manner similar to LPS. *A*, Mouse primary osteoblasts and bone marrow (BM) cells were cocultured with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) or OPG (100 ng/ml). TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures. *B*, Primary osteoblasts and bone marrow cells were cultured separately with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) for 6 h. The PGE$_2$ concentration in the culture supernatant was determined using EIA. Values are expressed as the means ± SD of quadruplicate cultures. *C*, Primary osteoblasts were treated with IL-1β (10 ng/ml) for 3 h, and then COX2 and β-tubulin mRNA expression was analyzed by Northern blotting. Figures below the signals represent the intensity of the COX2 mRNA signals relative to the β-tubulin mRNA signals. *D*, Primary osteoblasts prepared from OPG$^{-/-}$ mice and wild-type bone marrow cells were cocultured with IL-1β (10 ng/ml) in the presence or absence of NS398 (1 μM) or OPG (100 ng/ml). TRAP-positive cells containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SD of quadruplicate cultures.

This suggests that LPS induces RANKL expression by the mechanism independent of PGE$_2$ production. Kikuchi et al. (40) reported that LPS induces RANKL through extracellular signal-regulated kinase (ERK) and PKC. We also confirmed that calcium/PKC inhibitors, such as BAPTA-AM (an intracellular calcium chelator) and Ro-32-0432 (a PKC inhibitor), and ERK inhibitor PD98059 inhibited LPS-induced RANKL mRNA expression in osteoblasts (K.S., unpublished observation). PD98059 failed to inhibit the induction of RANKL mRNA expression by PGE$_2$ and the induction of osteoclast formation in cocultures treated with PGE$_2$ (data not shown). These results suggest that LPS directly stimulates RANKL expression through calcium/PKC signals, followed by ERK signals in osteoblasts. It is unlikely that PGE$_2$-induced signals directly cross talk with LPS-induced signals in the induction of RANKL expression in osteoblasts.

The intracellular signaling pathway of TLR4 is quite similar to that of IL-1Rs (25–29). Like LPS, IL-1 stimulated COX2 mRNA expression at 3 h and PGE$_2$ production at 6 h in osteoblast cultures (Fig. 5). IL-1β also induced RANKL mRNA expression in osteoblasts, as previously reported (data not shown) (32). NS398 inhibited IL-1-induced osteoclast formation strongly in cocultures containing wild-type osteoblasts, but only partially in cocultures containing OPG$^{-/-}$ osteoblasts (Fig. 5). These results suggest that PGE$_2$ produced by osteoblasts in response to IL-1 plays a similar role to LPS in osteoclast formation through the suppression of OPG expression (Fig. 6).

In cocultures containing OPG$^{-/-}$ osteoblasts, osteoclasts were formed even in the absence of any stimulus (Fig. 4). The spontaneous osteoclast formation was strongly inhibited by the addition

of either OPG or NS398 (Fig. 4). These results suggest that RANKL is involved in the spontaneous osteoclast formation, and that PGE$_2$ constitutively produced in the cocultures stimulates RANKL expression in osteoblasts. LPS and IL-1β further enhanced osteoclast formation in cocultures containing OPG$^{-/-}$ osteoblasts (Fig. 4), suggesting that the up-regulation of RANKL expression by LPS and IL-1 enhances the osteoclast formation. The induction of osteoclast formation by LPS and IL-1 in cocultures containing OPG$^{-/-}$ osteoblasts was partially inhibited by the addition of NS398. Therefore, PGE$_2$ induced by LPS and IL-1 appears to be involved in RANKL expression in osteoblasts. Our results indicate that the full inhibition of LPS- and IL-1-induced osteoclast formation by NS398 requires PGE$_2$-dependent suppression of OPG production (Fig. 6).

The previous studies have shown that OPG production by osteoblasts is down-regulated by bone-resorbing factors such as 1,25(OH)$_2$D$_3$, PTH, and PGE$_2$ (38, 41–44). Our results confirmed the previous finding that osteoclasts spontaneously form in the control cocultures containing OPG$^{-/-}$ osteoblasts. The decrease in OPG production by osteoblasts was a key event for the induction
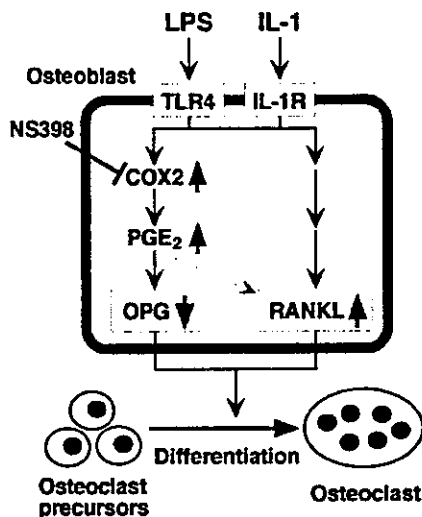
FIGURE 6. A possible mechanism of the induction of osteoclastogenesis by LPS and IL-1. LPS and IL-1 promote the differentiation of osteoclast precursors into osteoclasts through two parallel events in osteoblasts: direct enhancement of RANKL expression, and suppression of OPG production mediated by PGE_2. PGE_2 induced by LPS and IL-1 also stimulates RANKL expression, but the suppression of OPG production in osteoblasts appears to be more important than the induction of RANKL expression in osteoblasts for the stimulation of osteoclastogenesis. See text for details.

of osteoclastogenesis by LPS and IL-1. $OPG^{-/-}$ mice exhibited severe osteoporosis caused by enhanced osteoclast formation and function (14, 15, 45, 46). These results suggest that OPG is a physiological regulator of bone resorption, and that the balance between RANKL and OPG expressions at bone is particularly important for the regulation of bone resorption in vivo and in vitro.

We previously reported that LPS and IL-1 directly stimulated the survival, fusion, and pit-forming activity of osteoclasts (47). Those results together with the results shown in this study suggest that LPS and IL-1 are involved in the stimulation of osteoclastic bone resorption in several ways: LPS and IL-1 directly stimulate osteoclast function, induce RANKL expression in osteoblasts, and suppress OPG expression through enhancement of PGE_2 production. Further studies will be necessary to elucidate the precise mechanism of the regulation of osteoclastic bone resorption induced by these inflammatory factors.

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