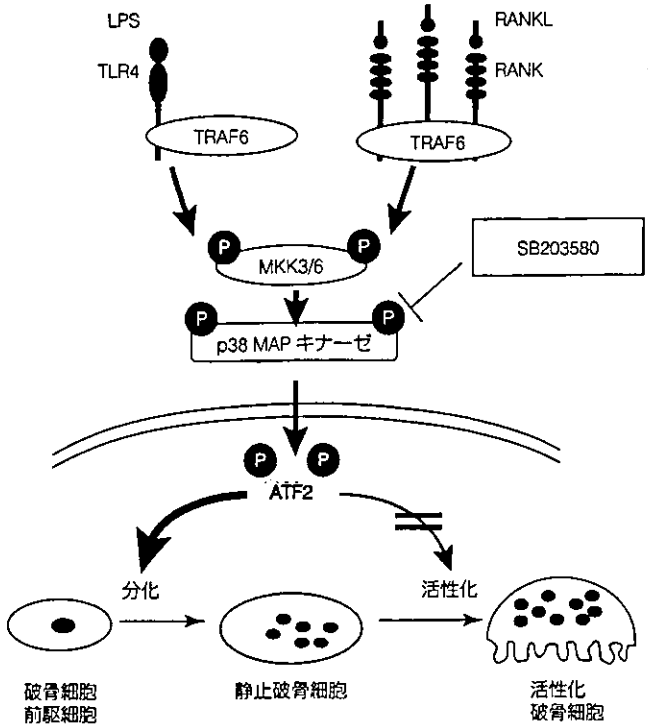

図 3. p38 MAP キナーゼシグナルの破骨細胞分化における重要性
p38 MAP キナーゼのリン酸化は破骨細胞の分化の必須シグナルであるが，分化した破骨細胞においては p38 のシグナル伝達系が作動しないように調節されている．

RANKL mRNA 発現を時間依存的に上昇させた．この発現上昇は NS 398(シクロオキシゲナーゼ 2 阻害剤)により抑制され，共存培養における破骨細胞分化も完全に阻害された[27]．

前述のように，LPS は破骨細胞に直接作用することにより，成熟破骨細胞の延命を促進する．この LPS による破骨細胞の延命効果が TLR 4 を介しているかどうかを検討するために，TLR 4 に点変異を有する LPS 低反応性の C 3 H/HeJ マウスを用いて実験をおこなった．C 3 H/HeJ マウスおよびコントロールマウスとして C 3 H/HeN マウス由来の骨髄細胞から誘導したそれぞれの破骨細胞における IL-1，LPS の延命効果について検討した．その結果，IL-1 処理による延命効果は C 3 H/HeJ，C 3 H/HeN 両マウスにおいて効果が認められたが，LPS の延命効果は C 3 H/HeJ マウスではまったく認められなかった．また，LPS による破骨細胞の延命効果は，OPG または IL-1 中和抗体の添加によって抑制されなかった．さらに，TNF レセプター欠損マウスから誘導した破骨細胞を用いた実験では LPS は RANKL と同様に延命効果が認められた[28]．

LPS のマクロファージに対する作用として，各種炎症性サイトカインの産生がある．そこで，単核マクロファージから分化する破骨細胞におけるサイトカインの産生を検討した．破骨細胞を含む細胞群に LPS を 24 時間処理した後，IL-1 β の特異抗体を用いた免疫染色をおこなったところ，単核マクロファージは IL-1 β の発現が認められたが，多核破骨細胞においては IL-1 β の発現はまったく認められなかった[28]．さらに，それぞれの細胞の培養上清中における IL-1 β，TNF および

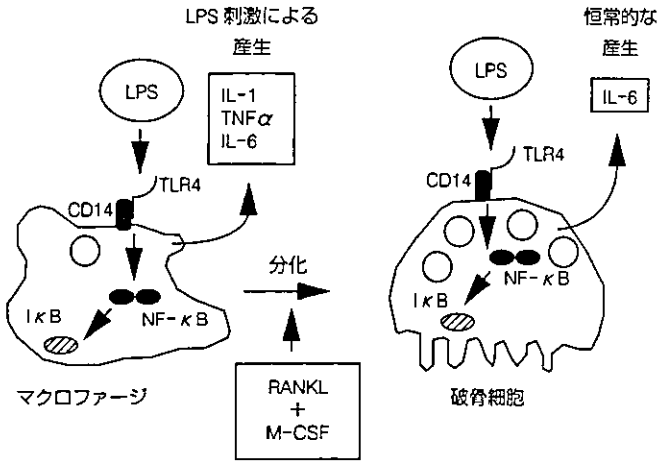

図 4. LPS(リポ多糖)の破骨細胞分化と機能発現に対する作用
破骨細胞の分化に対して，LPS は RANKL 発現を介する促進作用を示す．また，成熟破骨細胞においても破骨細胞前駆細胞(マクロファージ)と同様に LPS レセプターが認められ，LPS が破骨細胞の TLR 4 を介して直接その延命を促進する．一方，マクロファージにみられる LPS 刺激によるサイトカイン産生誘導は破骨細胞では認められない．

IL-6 の分泌について ELISA 法を用いて検討した．その結果，IL-1β と TNF の両サイトカインは，腹腔マクロファージと骨髄マクロファージにおいて LPS の処理後に強い産生誘導が認められたが，破骨細胞による産生はまったく認められなかった．破骨細胞においては，IL-6 の産生促進は恒常的に認められ，マクロファージにみられる LPS による産生誘導は認められなかった[28]．

以上をまとめると，破骨細胞の分化に対して，LPS は RANKL 発現を介する促進作用を示すことが明らかになった．また，成熟破骨細胞においても LPS レセプターが認められ，LPS が破骨細胞の TLR 4 を介して直接その延命を促進していることが示された(図 4)．一方，マクロファージにみられる LPS 刺激によるサイトカイン産生誘導は破骨細胞ではまったく認められなかった．このように破骨細胞はマクロファージ由来の細胞でありながら，サイトカイン産生能を有さない非炎症性細胞に分類されると考えられる．この LPS に対する両細胞の反応性の差異は，p 38 MAP キナーゼのシグナル伝達の差異に起因する可能性も考えられる．

5．RANK-RANKL 系を介さない TNFα の破骨細胞形成促進機構

マウス骨髄細胞を M-CSF の存在下で 4 日間培養して得たマクロファージを RANKL と M-CSF の存在下でさらに 3 日間培養すると，大部分のマクロファージは破骨細胞に分化する[23]．そこでこの培養系を用いて各種サイトカインの作用を調べたところ，マウス TNFα は M-CSF の存在下でマクロファージからの破骨細胞への分化を促進した．一方，ヒト TNFα はわずかな数の単核破骨細胞を誘導するのみであった．このマクロファージの単独培養系において，IL-1 には破骨細胞の分化を誘導する活性は認められなかった．また，マウス TNFα による破骨細胞形成は OPG の添加によりまったく抑制されなかったが，TNF Ⅰ型レセプターならびに TNF Ⅱ型レセプターに対する中和抗体によって強力に抑制された[23]．マ

ウスTNFαはマウスのTNF I型レセプターとII型レセプターに結合しシグナルを伝達するのに対し，ヒトTNFαはマウスのTNF I型レセプターにのみ結合する．以上の知見は，TNF I型レセプターおよびII型レセプター両者からのシグナルが破骨細胞の分化に重要であることを示唆するものである．また，マクロファージの形質を有するマウス細胞株であるRAW 264.7細胞の単独培養においてもTNFα刺激によって破骨細胞の形成が認められた[29][30]．TNFαのシグナル伝達にはTRAF 2が必須であることが報告されており，TNFα誘導性の破骨細胞形成におけるTRAF 2の重要性が示唆される．しかし，TRAF 6遺伝子欠損マウスから得られた破骨細胞前駆細胞にTNFαを処理してもRANKLの場合と同様に破骨細胞はほとんど形成されないことから[31]，TNFαによる破骨細胞形成におけるTRAF 2とTRAF 6の関連は今後に残された課題である．

つぎに，TNFαによって誘導された破骨細胞に骨吸収活性が具備されているか否かを解析した．マウスTNFαとM-CSFの存在下で破骨細胞前駆細胞を象牙切片上で培養すると，破骨細胞は誘導されたが吸収窩は形成されなかった．吸収窩は，TNFαとIL-1を同時に添加したときにのみ象牙切片上に形成された[23]．以上の知見より，マウスTNFαは破骨細胞の分化を促進するが，破骨細胞の活性化を誘導しないこと，一方IL-1は，破骨細胞前駆細胞から破骨細胞への分化を直接促進しないが，形成された破骨細胞の骨吸収活性を誘導することが明らかにされた．このことから，破骨細胞の機能発現にはTRAF 6が必須であることがわかる．

Pacifici ら[32][33]は，閉経後のエストロゲン欠乏はTリンパ球によるTNFαの産生亢進を介して骨吸収亢進を惹起するという実験結果を報告した．すなわち，卵巣摘出術をおこなったマウス骨髄においてはTNFを産生するTリンパ球の数が有意に増加すること，Tリンパ球が欠如しているヌードマウスあるいはTNF I型レセプター遺伝子欠損マウスにおいては卵巣摘出術による骨量減少が認められないという興味深い結果である．以上の結果は，エストロゲン欠乏による骨破壊にもTリンパ球によるTNF産生が密接に関与している可能性を示している．

おわりに

1997年のOPGの発見とそれにつづく1998年のRANKL遺伝子のクローニングにより，破骨細胞の形成を調節する骨芽細胞の役割の詳細が明らかになりつつある．さらに，RAや歯周疾患の発症に関与するさまざまな炎症性サイトカインとRANKLとのシグナル伝達の複雑なクロストークのベールもはがされつつある．また，RANKLとRANKの遺伝子欠損マウスが作製され，これらのマウスがともに大理石骨病を呈することが示され，少なくとも生理的骨吸収ではRANKを介するシグナル伝達系が主要な役割を果たすことが示唆されている．一方，炎症性サイトカインであるTNFαとIL-1はRANKL系を介さずにそれぞれ破骨細胞の分化と機能を促進する．とくに，TNFαについては，リウマチ患者に対するTNF抗体の投与が関節破壊を著明に改善したという臨床知見が欧米とわが国であいついで報告されている．したがって，RAや歯周疾患をはじめとする炎症性骨吸収の亢進にはTNFをはじめとする炎症性サイトカインの関与が示唆される．

この数年にわたる骨吸収機構に及ぼす各種新規サイトカインの発見は，破骨細胞による骨吸収の仕組みの分子レベルでの理解に大きく貢献した．これらの研究の発展が歯周病の新しい治療方針の確立や治療薬の開発につながることを期待したい．

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2. 破骨細胞の形成と機能を調節する炎症性サイトカインとリポ多糖（LPS）の作用機構

小林泰浩，宇田川 信之，高橋直之

骨芽細胞が破骨細胞の分化や機能を調節することは従来知られていたが，receptor activator of NF-κB ligand（RANKL）の発見により，その調節機構を分子レベルで説明することが可能となった．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の発現を促し，破骨細胞の分化と活性化を誘導する．TNFα，IL-1など炎症性サイトカインやリポ多糖などの細菌菌体成分も破骨細胞の分化と機能を調節していることが判明し，炎症性骨吸収の発症機構が明らかにされつつある．

はじめに

骨吸収は単球・マクロファージ系の前駆細胞より分化した破骨細胞によって担われる．この破骨細胞の分化と機能発現は，骨形成を司る骨芽細胞・骨髄由来間質細胞（本稿では両細胞群を骨芽細胞とする）により厳密に調節されている[1]．1998年，骨芽細胞が発現する破骨細胞の分化と機能を調節する破骨細胞分化因子（osteoclast differentiation factor：ODF）がクローニングされ，骨吸収調節メカニズムの一端が分子レベルで明らかにされた[2]．このODFはTNF（tumor necrosis factor，腫瘍壊死因子）ファミリーに属する新規のサイトカインで，Choiらのグループ[3]によって報告されたTRANCE（TNF-related activation-induced cytokine），あるいはImmunexのグループ[4]によって報告されたRANKL（receptor activator of NF-κB ligand）と同一分子であった．以来，免疫学の主役と思われていたサイトカインのファミリーが直接破骨細胞の形成や機能を制御する知見も集積されてきた．近年，とりわけ破骨細胞の分化と活性化を修飾する炎症性サイトカインや細菌由来の成分の役割が注目されている．本稿では，破骨細胞の分化と活性制御の分子メカニズムとともに，炎症性サイトカインと細菌菌体成分の骨吸収調節機構について概説したい．

[キーワード&略語]
破骨細胞，骨芽細胞
RANKL：receptor activator of NF-κB ligand
OPG：osteoprotegerin
TLR：Toll-like receptor
TRAF：TNF receptor associated factor
IL-1：interluekin-1
PAMP：pathogen-associated molecular patterns
LPS：lipopolysaccharide

1 TNFα/IL-1による破骨細胞の分化と活性化の制御

TNFαは破骨細胞の前駆細胞である骨髄由来マク

Regulation of osteoclast differentiation and function by proinflammatory cytokines and pathogen-associated molecular patterns
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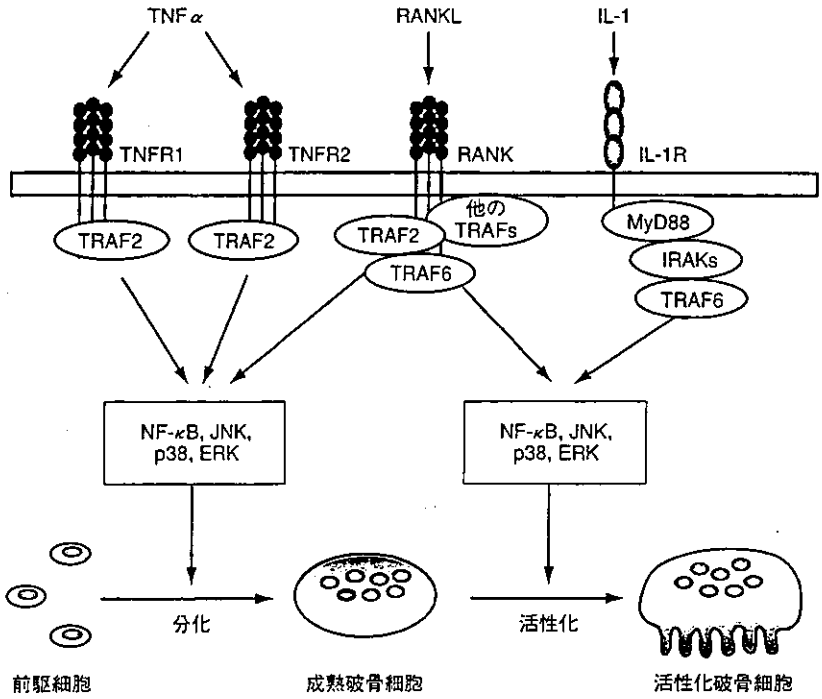

図1　破骨細胞の分化と機能発現を誘導するTNFレセプターファミリーおよびIL-1レセプターのシグナル伝達
TNFαがTNFレセプターに結合するとその下流に存在するTRAF2が活性化される．同様にRANKでは，TRAF1, TRAF2, TRAF3, TRAF5, TRAF6が活性化される．一方，IL-1レセプターでは，TRAF6が活性化される．それぞれのレセプターによって活性化されたTRAFはJNK, p38MAPK, NF-κB, ERKを活性化し，破骨細胞の分化および機能発現が誘導される．これらのTRAFのうち，TRAF6を介するシグナルが破骨細胞の分化と機能発現の誘導に重要であることを最近の知見は示唆している

ロファージに直接作用し破骨細胞への分化を誘導する[5)6)]．このTNFαによる破骨細胞形成促進作用はRANKLのデコイレセプターであるosteoprotegerin（OPG）によって抑制されず，TNF I型ならびにII型レセプターに対する中和抗体によって強力に抑制された．また，TNFαはマクロファージ系株化RAW264.7細胞の破骨細胞への分化をRANKL非存在下で促進する[7)]．これらの知見は，TNFαはRANKL-RANKシステムを介さずに破骨細胞分化を誘導することを示唆する．一方，IL-1（interleukin-1）は破骨細胞の分化を誘導しないが，RANKLと同様に成熟破骨細胞に直接作用し，その骨吸収活性を誘導することが示された[8)]．

TNFファミリーに属するサイトカインのシグナルは，それぞれのレセプターの細胞内ドメインに会合するTNF receptor-associated factor（TRAF）を介して伝達される．それぞれのレセプターには，異なったパターンでTRAFが会合する．TNFレセプターにはTRAF2が，RANKLのレセプターであるRANKにはTRAF1, TRAF2, TRAF3, TRAF5, TRAF6が会合する[9)]．一方，IL-1はTNFファミリーには属さないが，IL-1レセプターを介するシグナルはTRAF6を活性化する．以上の知見から，破骨細胞の分化にはTFAF2を介するシグナルが，そして破骨細胞の活性化にはTRAF6を介するシグナルが重要であると思われる（図1）．

一方，TRAF6欠損マウスは典型的な大理石骨病を呈することが報告され，骨吸収を調節するTRAF6のシグナルの重要性が注目されている[10)11)]．TRAF6欠損マウスから得られた破骨細胞前駆細胞はRANKLあるいはTNFαによっても破骨細胞への分化が著しく抑制されているという．また，TNFαが誘導する破骨細胞形成を少量のRANKLが強く促進することも報告された[12)]．さらに最近，Yeら[13)]は，TRAF6によるレ

セプター細胞内領域の認識機構が他のTRAFとは大きく異なること，TRAF6と強い親和性をもつRANK細胞内領域由来の細胞透過性デコイペプチドは，RANKLによる破骨細胞分化を著明に抑制することを明らかにした．これらの知見は，破骨細胞の分化誘導にもTRAF6からのシグナルがきわめて重要であることを示唆するものである（図1）．骨吸収に関与するRANKL, IL-1, TNFαなどサイトカイン間におけるシグナル伝達系のクロストークの解明が，今後急速に進むと思われる．

表1　マウスのTLRとそれらが認識する細菌由来因子

TLR	リガンド	参考文献
TLR1	表層タンパク質A（OspA）	15
TLR2	ペプチドグリカン（PGN），リポタンパク質，リポペプチド，リポアラビノマンナン，OspA	14
TLR3	細菌二本鎖RNA（dsRNA），ポリI：C	14
TLR4	リポ多糖（LPS），リポタイコ酸（LTA），タキソール，熱ショックタンパク質60	14
TLR5	フラジェリン	14
TLR6	？	14
TLR7	免疫賦活剤イミダゾキノリン類（imiquimod, R-848）	16
TLR8	？	14
TLR9	細菌CpG DNA	14

2 細菌性抗原分子を認識するToll様レセプターとそのシグナル

近年，マクロファージや樹状細胞は細菌を構成する抗原性分子（pathogen-associated molecular patterns：PAMP）をToll様レセプター（Toll-like receptor：TLR）を介して認識し，さまざまな免疫反応を起こすことがわかってきた[14]．TLRはショウジョウバエにおいて真菌の感染防御に関与するTollのホモログであり，ヒトで10種類，マウスで9種類知られている．これらTLRが認識する細菌を構成する抗原性分子もこの数年間で次々と明らかにされた（表1）[14)~16)]．TLRに関する知見をまとめると，①TLRはそれぞれ特異的な細菌細胞成分と結合し，シグナルを細胞内に伝達する．②TLRの細胞内ドメインはIL-1レセプター（IL-1R）ファミリーの属するレセプターのそれと類似し，ともにMyD88とIRAK（IL-1 receptor-associated kinase），さらにTRAF6を介してシグナルが伝達される．③TRAF6を介するシグナルは，さらにMAPK（mitogen activated protein kinase）の活性化やNF-κB, AP-1の活性化を誘導する．④TLR4からのシグナルはMyD88依存性経路とともにTIRAP（Toll-IL-1 receptor domain-containing adapter protein）を介するMyD88非依存性経路がある．このMyD88非依存性経路はTLR4以外の他のTLR/IL-1Rには認められない[17]．

このように，この数年間でLPSをはじめとするPAMPの受容機構とシグナル経路が急速に明らかにされた[14]．また，PAMPの破骨細胞前駆細胞と破骨細胞に対する効果も詳細に解析されつつある．

3 PAMPの破骨細胞前駆細胞と破骨細胞に対する直接作用

破骨細胞の前駆細胞である骨髄マクロファージは，TLR1からTLR9まですべてのTLRを発現している[18]．骨髄マクロファージはRANKLとM-CSF（macrophage colony-stimulating factor）の存在下で培養すると破骨細胞に分化する．高見ら[18]はLPS，ペプチドグリカン，二本鎖RNA，ポリI：C，CpG DNAなどPAMPは破骨細胞前駆細胞に作用し，RANKLとM-CSFが誘導する破骨細胞分化を著しく抑制することを報告した．この知見は，菌体成分はマクロファージから破骨細胞への分化を抑制し，骨組織への細菌侵入の防御に働いている可能性を示している．一方，IL-1は骨髄マクロファージのp38MAPKを活性化するにもかかわらず（IL-1レセプターは機能するにもかかわらず）[16) 19)]，RANKL誘導性の破骨細胞形成を全く抑制しない[18]．IL-1RとTLRのシグナル伝達系の類似性を考えると，破骨細胞分化に対するIL-1とPAMPの作用の違いは大変に興味深い．

成熟破骨細胞に対するPAMPの作用も解析された[18) 20)]．成熟破骨細胞はTLR2とTLR4のmRNAを発現しているが，他のTLRは発現していない[18]．実際に，LPSとペプチドグリカンは，IL-1と同様に破骨細胞の延命を促進した[20) 21)]．また，LPSはIL-1と同様に破骨細胞の骨吸収活性を促進した．LPSによる

表2　正常およびOPG遺伝子欠損マウス由来の骨芽細胞と骨髄細胞を用いた共存培養系における破骨細胞形成に及ぼすNS398の効果

	破骨細胞誘導因子		
共存培養系	LPS	IL-1	1, 25 $(OH)_2D_3$
正常マウス	↓	↓	→
$OPG^{-/-}$マウス	→	→	→

骨芽細胞と骨髄細胞を用いた共存培養系にLPS, IL-1あるいは1, 25 $(OH)_2D_3$を添加して破骨細胞形成を促した．さらにCOX2の阻害剤NS398を添加し，LPS, IL-1あるいは1, 25 $(OH)_2D_3$が誘導する破骨細胞形成を評価した．NS398の効果は矢印で示した．↓：抑制，→：抑制しない

破骨細胞延命効果は，TLR4遺伝子に異常のあるC3H/HeJマウス由来の細胞では起こらないことから，LPSによる破骨細胞の延命促進作用はTLR4を介するものと推察される．以上のように，PAMPによる成熟破骨細胞の活性化および延命促進作用はTLR4もしくはTLR2を介する．

前述したように，TLR4からのシグナルにはMyD88依存性と非依存性の経路が存在する．また，IRAKのメンバーの1つでマクロファージ系の細胞が特異的に発現しているIRAK-Mの欠損マウスが作製された[22]．IRAK-M欠損マウス由来のマクロファージはTLRの刺激に対してサイトカイン産生が増強する．また，IRAK-M欠損マウスは細菌感染に対して過剰な免疫反応が起こることが示され，IRAK-MはTLRのシグナルを負に調節する因子であることが明らかになった．TIRAPやIRAK-Mのように，シグナルを別の経路で伝達する分子や負に調節する分子が，LPSとIL-1の破骨細胞形成調節作用の違いに関与している可能性も考えられる．今後の研究で，LPSとIL-1の作用の違いが明確化されることが期待される．

4 LPSとIL-1の破骨細胞誘導作用：OPG発現抑制の重要性

マウス骨髄細胞と骨芽細胞の共存培養系において，破骨細胞形成に及ぼすLPSならびにIL-1の作用を検討したところ，LPSとIL-1は破骨細胞形成を促進した．実際にLPSとIL-1は骨芽細胞のRANKLの発現を促進し，OPGの発現を抑制した[23]．また，Cox 2 (cyclooxygenase 2，誘導型プロスタグランディン合成酵素）の阻害薬であるNS398は活性型ビタミンD [1, 25 $(OH)_2D_3$] が誘導する破骨細胞形成は抑制しなかったが，LPSとIL-1による破骨細胞の形成を完全に阻害した．このことから，LPSとIL-1はPGE_2の産生を介して，RANKLの発現亢進とOPGの発現抑制を惹起するものと推測された．さらに，OPG遺伝子欠損マウス由来の骨芽細胞と骨髄細胞を用いた共存培養系におけるLPSとIL-1による破骨細胞形成が解析された（表2）．興味深いことに，OPG欠損マウスから得られた細胞を用いた共存培養において，NS398はLPSとIL-1による破骨細胞形成を全く抑制しなかった（表2）[23]．この共存培養系にOPGを添加するとLPSによる分化が完全に抑制された．

さらに，正常マウスの骨芽細胞培養系で，NS398は1, 25 $(OH)_2D_3$とともにLPSとIL-1によるRANKLの発現誘導を全く阻害しなかった（表3）．また，恒常的に発現しているOPG mRNAは，1, 25 $(OH)_2D_3$, LPSあるいはIL-1L処理によって減少した．NS398をさらに添加するとLPSあるいはIL-1Lによって誘導されたOPGの発現抑制のみが回復した（表3）．これらの知見は，共存培養系でのLPSとIL-1による破骨細胞形成は，次の2つの事象により引き起こされることを示す．第一にLPSとIL-1はRANKLを誘導するが，このRANKL誘導にPGE_2は全く関与しない．第二にLPSとIL-1はPGE_2の産生亢進を介してOPGの発現を抑制する．このOPGの発現抑制はLPSとIL-1による破骨細胞形成にとってきわめて重要である．NS398の抑制効果は，OPG欠損マウスでは認められない．すなわち，LPSまたはIL-1は骨芽細胞に作用し直接RANKLの発現を誘導するとともに，PGE_2の産生を介してOPG産生を抑制し破骨細胞の分化を誘導するものと考えられた．

前述したように，骨髄マクロファージの培養系にLPSなどPAMPを添加すると，RANKLとM-CSFによって誘導される破骨細胞形成は強力に抑制される．

表3　マウス骨芽細胞における各種刺激因子によるRANKLとOPGの発現調節とPGE_2の役割

刺激因子	遺伝子の発現とPGE_2の関与	
	RANKL	OPG
LPS	↑（PGE_2非依存性）	↓（PGE_2依存性）
IL-1	↑（PGE_2非依存性）	↓（PGE_2依存性）
1, 25 $(OH)_2D_3$	↑（PGE_2非依存性）	↓（PGE_2非依存性）

骨芽細胞をLPS, IL-1あるいは1, 25 $(OH)_2D_3$で処理しmRNAの発現を解析した．さらにCOX2阻害剤であるNS398を添加し，RANKLとOPG遺伝子の発現に対するPGE_2の役割を検索した．mRNAの発現に対する効果は矢印で示した（↑：促進，↓：抑制）．NS398の添加効果は「PGE_2依存性」と「PGE_2非依存性」として表した．PGE_2非依存性：NS398は何らmRNAの発現に影響を与えなかった．PGE_2依存性：NS398はmRNAの発現に及ぼす刺激因子の作用を抑制した

しかし，共存培養系においては，PAMPは破骨細胞形成を促進する．この矛盾は現段階では説明できないが，骨芽細胞は破骨細胞前駆細胞におけるPAMPのシグナル（破骨細胞への分化を抑制する）を中和する因子を産生しているのかもしれない．

5 LPSとIL-1のRANKL誘導作用

1, 25 $(OH)_2D_3$, PTH（parathyroid hormone，副甲状腺ホルモン），PGE_2，IL-11等の骨吸収促進因子は骨芽細胞に作用してRANKLの発現を誘導する（図2）[24]．1, 25 $(OH)_2D_3$はビタミンDレセプター（VDR）を介して，IL-11やIL-6はgp130を介してRANKLの発現を促進する．一方，PTHとPGE_2によるRANKLの発現上昇はcAMP/PKA系が仲介すると考えられる．また，骨芽細胞をイオノマイシンであるA23187で処理したり，細胞外Ca^{2+}濃度を上昇させたりすると，PKC（protein kinase C）の活性化を介してRANKLの発現が誘導される．このように，骨芽細胞におけるRANKL遺伝子の発現は，少なくとも4つの独立したシグナル系（VDR, gp130, cAMP/PKA, Ca^{2+}/PKC）により調節されている．それでは，前述したLPSとIL-1によるRANKL発現誘導作用はどのようなシグナル系を介するのであろうか．

LPSとIL-1は骨芽細胞のRANKL mRNAの発現を亢進するが，この発現誘導はNS398では抑制されず，PKC阻害剤であるRO-31-8220とERKの阻害剤であるPD98059で特異的に阻害される[21)25)]．また，A23187やPKCの賦活剤であるPMA（phorbol 12-myristate 13-acetate）はERK（extracellular signal regulated kinase）/MEK（MAPK/ERK kinase）の活性化（リン酸化）を誘導し，この誘導をPD98059は阻害する．これらの知見は，①細胞内Ca^{2+}濃度の上昇によるPKCの活性化によって誘導されるRANKLの発現は，MEK/ERK系シグナルを介すること，②TLR/IL-1Rからのシグナルは PKCの活性化とそれに続くMEK/ERKを介したシグナル伝達を介してRANKLを誘導することを示唆するものである（図2）．

おわりに

'97年のOPGの発見とそれに続くRANKLのクローニングにより，破骨細胞の形成と分化を調節するシグナル伝達が分子レベルで説明できるようになった．また，リウマチや炎症時に産生されるサイトカインは骨芽細胞のRANKLの発現を促進するのみならず，破骨細胞前駆細胞や成熟破骨細胞へ直接作用し，活性化や延命を調節する機構もわかってきた．さらに，'97年細菌の菌体成分を認識するレセプターのヒトおよびマウスのホモログ（TLR）が同定されて以来，菌体成分による骨吸収調節機構が急速に解明されつつある．破骨細胞の分化と活性化における炎症性サイトカインと菌体成分からのシグナル系の*in vitro*と*in vivo*両面での詳細な解析は，炎症性骨吸収の治療薬の開発や治療指針の確立に大いに貢献するであろう．

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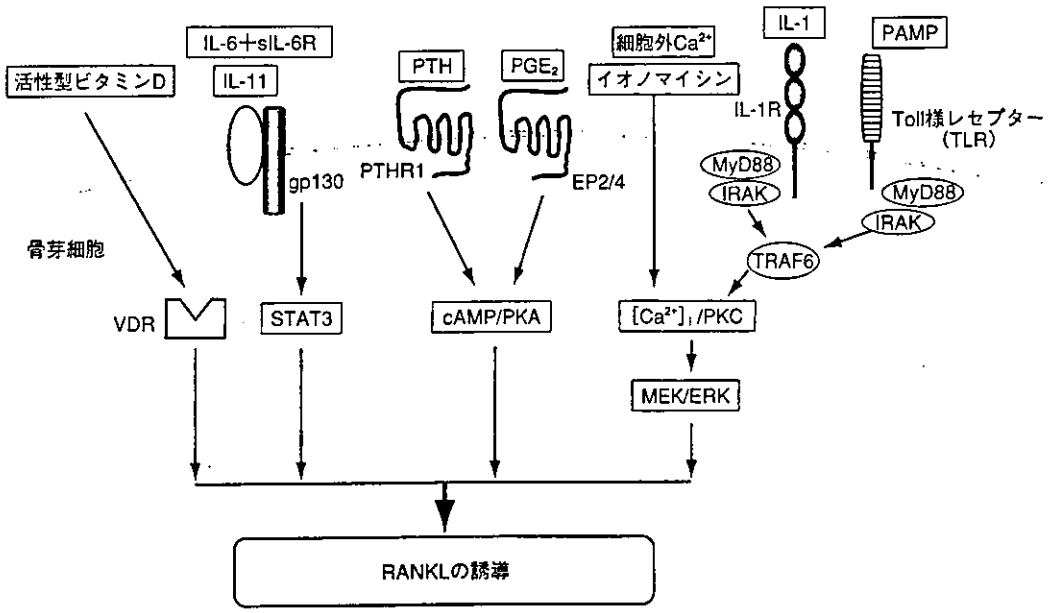

図2　骨芽細胞におけるRANKL誘導のシグナル伝達系：LPSとIL-1はPKC-MEK/ERK系を介してRANKLを誘導する

骨芽細胞におけるRANKL遺伝子は，少なくとも4つの独立したシグナル系を介して発現誘導される．1, 25 $(OH)_2D_3$はVDRを介して，IL-11やIL-6はgp130を介して，PTHとPGE_2はcAMP/PKAを介してRANKL遺伝子の発現を促進する．さらに，$[Ca^{2+}]$/PKCの活性化もRANKLの発現を誘導する．一方TLRとIL-1Rの細胞内領域は相同性が高く，共通の細胞内分子を介してシグナルを伝える．TLRとIL-1Rのシグナルも RANKLの発現を誘導するが，このシグナル系は $[Ca^{2+}]$/PKCを介する．さらに $[Ca^{2+}]$/PKC系はMEK/ERK系を介してRANKLを誘導する．これらのシグナルは共存培養系で破骨細胞の形成を支持するものである

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REVIEW ARTICLE
Bone Biology

Regulatory mechanisms of osteoblast and osteoclast differentiation

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Bone is continuously destroyed and reformed to maintain constant bone volume and calcium homeostasis in vertebrates throughout their lives. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Recent developments in bone cell biology have greatly changed our conceptions of the regulatory mechanisms of the differentiation of osteoblasts and osteoclasts. Bone morphogenetic proteins (BMPs) play critical roles in osteoblast differentiation. The discovery of Smad-mediated signals revealed the precise functions of BMPs in osteoblast differentiation. Transcription factors, Runx2 and Osterix, are found to be essential molecules for inducing osteoblast differentiation, as indicated by the fact that both Runx2-null mice and Osterix-null mice have neither bone tissue nor osteoblasts. Smad transcriptional factors are shown to interact with other transcription regulators, including Runx2. Also, the recent discovery of receptor activator of NF-κB ligand (RANKL)–RANK interaction confirms the well-known hypothesis that osteoblasts play an essential role in osteoclast differentiation. Osteoblasts express RANKL as a membrane-associated factor. Osteoclast precursors that express RANK, a receptor for RANKL, recognize RANKL through the cell–cell interaction and differentiate into osteoclasts. Recent studies have shown that lipopolysaccharide and inflammatory cytokines such as tumor necrosis factor receptor-α and interleukin 1 directly regulate osteoclast differentiation and function through a mechanism independent of the RANKL–RANK interaction. Transforming growth factor-β super family members and interferon-γ are also shown to be important regulators in osteoclastogenesis. These findings have opened new areas for exploring the molecular mechanisms of osteoblast and osteoclast differentiation.
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Introduction

Bone is continuously destroyed and reformed in vertebrates in order to maintain bone volume and calcium homeostasis throughout their lives. Osteoblasts and osteoclasts are specialized cells responsible for bone formation and resorption, respectively. Osteoblasts produce bone matrix proteins including type I collagen, the most abundant extracellular protein of bone, and also take charge of mineralization of the tissue (Aubin and Triffitt, 2002). Osteoblasts, chondrocytes, myocytes and adipocytes are all derived from a common progenitor called undifferentiated mesenchymal cells. During the process of their differentiation, progenitor cells acquire specific phenotypes under the control of respective regulatory factors. Bone morphogenetic proteins (BMPs) play critical roles in the differentiation of undifferentiated mesenchymal cells into osteoblasts. Recent studies have elucidated the molecular mechanism of osteoblast differentiation induced by BMP.

Osteoclasts are multinucleated cells responsible for bone resorption. The most characteristic feature of osteoclasts is the presence of ruffled borders and clear zone (Väänänen and Zhao, 2002). Vacuolar H^+-ATPase exists in the ruffled border membrane of osteoclasts, and acidifies resorbing area under the ruffled border. The ruffled border is surrounded by a clear zone, which serves for the attachment of osteoclasts to the bone surface to maintain a microenvironment favorable for bone resorption. Osteoclasts are differentiated from hemopoietic cells of the monocyte/macrophage lineage

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under the control of bone microenvironments. Osteoblasts or bone marrow stromal cells have been shown to regulate osteoclast differentiation providing the microenvironment similar to bone. The recent discovery of new members of the TNF receptor-ligand family has clarified the molecular mechanism of osteoclast differentiation regulated by osteoblasts/stromal cells. This review article describes the current knowledge of the mechanisms of the regulation of osteoblast and osteoclast differentiation, which will deepen our understanding of oral biology and oral diseases.

Regulation of osteoblast differentiation

Characteristics of osteoblasts and their progenitors

Osteoblasts are specialized cells that function in bone formation in vertebrates. Bone tissue mainly consists of hydroxyapatite crystals and various kinds of extracellular matrix proteins including type I collagen, osteocalcin, osteonectin, osteopontin, bone sialoprotein and proteoglycans (Young *et al*, 1992; Robey *et al*, 1993; Mundlos and Olsen, 1997). Most of these bone matrix proteins are secreted and deposited by polarized mature osteoblasts, which are aligned on the bone surface. The formation of hydroxyapatite crystals in osteoid is also regulated by osteoblasts. Therefore, the expression of a number of bone-related extracellular matrix proteins, high enzyme activity of alkaline phosphatase (ALP), and responses to osteotropic hormones and cytokines are believed to be major characteristics of osteoblasts.

During embryogenesis, bone tissue is formed through two independent pathways: intramembranous ossification and endochondral ossification (Karsenty, 1999; Yamaguchi, komori and Suda, 2000). In both pathways, osteoblasts play unique roles in the bone formation. In the case of intramembranous ossification, osteoblasts are differentiated directly from mesenchymal cells in the mesenchymal condensation. On the other hand, in the endochondral ossification, mesenchymal cells differentiate into chondrocytes first and form a cartilaginous template. Then osteoblasts are differentiated from the surrounding mesenchymal cells immediately after maturation of hypertrophic chondrocytes in the template (Chung *et al*, 1998). These developmental processes of bone and cartilage suggest that osteoblasts and chondrocytes are derived from a common progenitor cell (Figure 1). Indeed, cell cultures prepared from calvaria or bone marrow show mixed populations of osteoblasts, chondrocytes, adipocytes and skeletal muscle cells. Some clonal embryonic fibroblast cell lines differentiate into multiple phenotypes of cells in response to treatment with 5-azacytidine (Taylor and Jones, 1979). The establishment of the pluripotent cell lines from the calvaria indicated that a pluripotent progenitor cell can differentiate into tissue-specific cells such as osteoblasts, chondrocytes, adipocytes and myoblasts (Grigoriadis, Heersche and Aubin, 1988, 1990; Yamaguchi and Kahn, 1991). The progenitor cells may acquire a tissue-specific phenotype concomitantly with losing their pluripotency under the control of various stimulants. Tissue-specific transcription factors regulate the differentiation of tissue-specific cells from the progenitor cells (Figure 1).

Discovery of BMPs

In 1965, Urist (1965) found that demineralized bone matrix contains a unique activity that induces ectopic bone when the matrix is implanted into muscular tissue. This activity was named 'BMP'. Subsequently, cDNAs encoding several active proteins for ectopic bone formation were isolated, and the proteins were eventually renamed 'BMPs' (Wozney *et al*, 1988). More than 15 genes of BMPs have been identified in vertebrates, and several recombinant BMP proteins have been shown to induce ectopic bone formation (Kingsley, 1994, 2001; Hogan, 1996; Wozney and Rosen, 1998; Reddi, 2001). Bone-inducing activity is unique to BMPs among the growth factors. It is believed that osteoblasts are cells responsible for the secretion and deposition of BMPs into the extracellular matrix during bone formation. BMPs, except BMP-1, belong to the transforming growth factor-β (TGF-β) superfamily, members of which are known to regulate the proliferation, differentiation and death of cells in various tissues (Hogan,

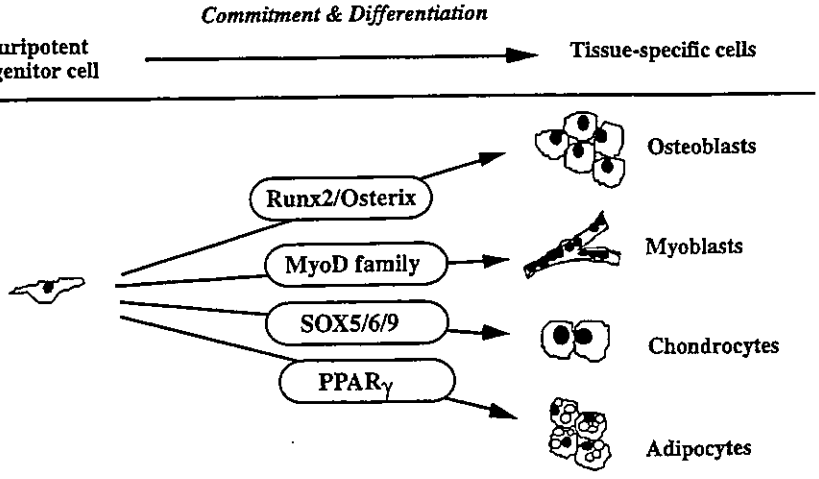

Figure 1 A schematic model for differentiation of a mesenchymal progenitor cell into tissue-specific cells. A pluripotent progenitor cell originated from undifferentiated mesenchyme can differentiate into several kinds of tissue-specific cells such as osteoblasts, myoblasts, adipocytes and chondrocytes. Each differentiation pathway seems to be regulated by tissue-specific transcription factors: Runx2/Osterix, MyoD family, PPARγ and SOX5/SOX6/SOX9, respectively

1996; Massague, 2000; Miyazono, ten Dijke and Heldin, 2000; Wrana, 2000). BMPs are also involved in the organogenesis of both hard and soft tissues (Kingsley, 1994, 2001; Hogan, 1996). Although TGF-β superfamily members have significant homology with each other, neither TGF-β nor activin/inhibin induces ectopic bone formation (Sampath, Muthukumaran and Reddi, 1987). BMPs are the only growth factors known at present to induce the whole process of ectopic bone formation in vertebrates.

Role of BMPs in skeletal development in vivo

The unique activity of BMPs suggests that they regulate osteoblast and chondrocyte differentiation during skeletal development. Identification of skeletal abnormalities in animals and patients with mutations in the BMP genes has confirmed this hypothesis. The first such example was the case of BMP-5 in mice (Kingsley *et al*, 1992). The mutant mouse 'short ear' has a defect in a gene required for normal growth and patterning of skeletal structures, and for repair of bone fractures in adults. Kingsley *et al* (1992) showed that the short ear region encodes BMP-5, which is deleted or rearranged in several independent mutations at the short ear locus. Storm *et al* (1994) reported that mutations in Gdf5, another member of the TGF-β superfamily, are responsible for skeletal alterations in brachypodism (bp) mice, which are characterized by skeletal abnormalities restricted to the limbs and limb joints. The human homolog of Gdf5, CDMP-1, has also been identified as a gene associated with a recessive chondrodysplasia, Hunter–Thompson type, which has a phenotype similar to that of bp mice (Thomas *et al*, 1996). Another mutation of CDMP-1 causes a chondrodysplasia of Grebe type, an autosomal recessive disorder characterized by more severe limb shortening and dysmorphogenesis than the Hunter–Thompson type (Thomas *et al*, 1997). In these patients, the mutated CDMP-1 protein shows a dominant-negative effect by preventing the secretion of other BMP members (Thomas *et al*, 1997). It has been suggested that overexpression of BMP-4 mRNA in human lymphocytes is associated with fibrodysplasia ossificans progressiva, a heritable disorder of connective tissue characterized by postnatal formation of ectopic bone in muscular tissues (Shafritz *et al*, 1996).

Other BMP-deficient mice have also been created, although some of them died at stages too early in development to examine their skeletal phenotypes. BMP-7-deficient mice have skeletal patterning defects restricted to the rib cage, skull and hindlimbs (Dudley, Lyons and Robertson, 1995; Luo *et al*, 1995). Homozygous mutant mice carrying a targeted deletion of Gdf11 (also called BMP-11) exhibit anteriorly directed homeotic transformations throughout the axial skeleton and posterior displacement of the hindlimbs (McPherron, Lawler and Lee, 1999). The skeleton of BMP-6 null mice is indistinguishable from that of wild-type mice, suggesting that BMP-2 may functionally compensate in BMP-6-null mice (Solloway *et al*, 1998). BMP-4/7 double heterozygotes develop minor defects in the rib cage and the distal parts of limbs (Katagiri *et al*, 1998). These findings clearly indicate that BMPs are key regulators of the differentiation of osteoblasts and chondrocytes during skeletal development. However, it is still unclear whether BMPs are involved in bone and cartilage formation after birth. Interestingly, BMP-3-null mice have twice as much trabecular bone after birth as wild-type littermates, suggesting that BMP-3 is a negative determinant of bone density (Daluiski *et al*, 2001).

Role of BMPs in osteoblast differentiation in vitro

In order to examine the molecular mechanism of the ectopic bone-induction, the biological effects of recombinant BMP proteins on osteoblast differentiation have been studied *in vitro* using cell lines and primary cells. In cultures of osteoblast lineage cells various BMPs enhance the expression of ALP, parathyroid hormone (PTH)/PTH-related protein (PTHrP) receptor, type I collagen, and osteocalcin (Yamaguchi *et al*, 2000). Furthermore, BMPs stimulated the formation of mineralized bone-like nodules (Yamashita *et al*, 1996). BMPs also induced osteoblast differentiation in some other types of cells in culture. C3H10T1/2 clone 8 (10T1/2), a cell line established from a C3H mouse embryo, differentiates into myoblasts, adipocytes and chondrocytes in the presence 5-azacytidine (Taylor and Jones, 1979). We and others showed that BMP-2 and BMP-4 induce osteoblast differentiation of 10T1/2 cells (Katagiri *et al*, 1990; Ahrens *et al*, 1993; Wang *et al*, 1993). BMPs also stimulate osteoblast differentiation of other pluripotent cell lines (Yamaguchi *et al*, 1991; Rosen *et al*, 1994).

Bone morphogenetic proteins were originally identified as an activity that induces an ectopic bone formation in muscular tissue, suggesting that BMPs regulate the pathway of differentiation of myogenic cells. To examine this possibility, we used a mouse myoblast cell line, C2C12. We found that BMP-2 inhibited myogenic differentiation of C2C12 myoblasts, and converted their differentiation pathway into that of osteoblasts (Katagiri *et al*, 1994). TGF-β1 also inhibited myogenic differentiation of C2C12 cells, but failed to induce osteoblast differentiation of the cells (Katagiri *et al*, 1994). Similar effects of BMPs were observed in primary myoblasts and other myogenic cell lines in culture (Katagiri *et al*, 1994; unpublished observations). It has also been reported that the combination of BMP-2 gene transfer by adenoviruses and orthotopic muscle grafting in rats resulted in the successful ossification of almost the whole grafted muscle (Gonda *et al*, 2000). C2C12 cells are believed to have been derived from satellite cells of muscular tissue (Yaffe and Saxel, 1977; Blau, Chiu and Webster, 1983). Satellite cells are a potential source of regenerating myoblasts *in vivo*. These results suggest that satellite cells in muscular tissue are potential progenitors which can differentiate into osteoblasts in response to BMPs.

BMP receptors

Signaling by TGF-β superfamily members, including BMPs, is basically initiated upon their binding to

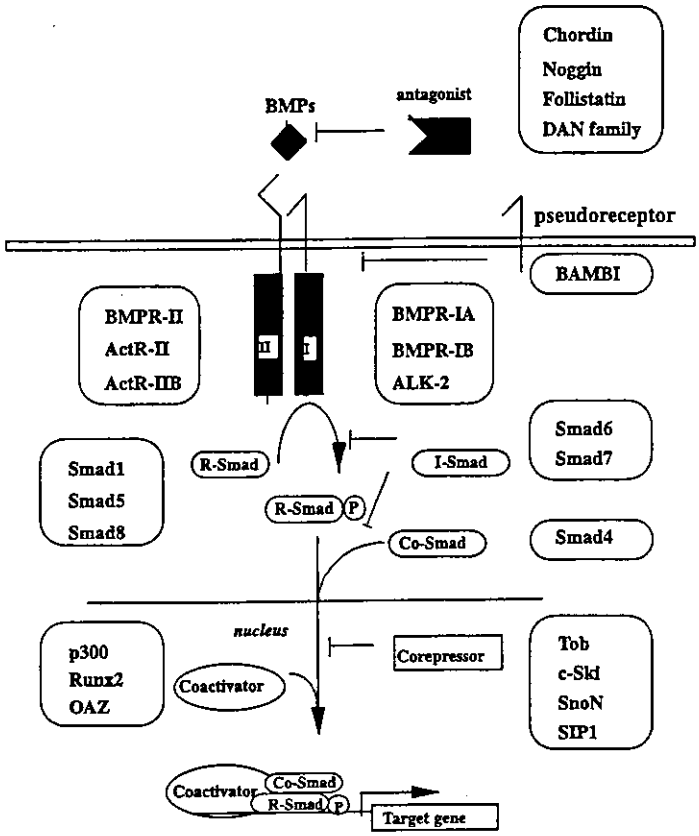

Figure 2 A schematic model for BMP signal transduction. Signaling of BMPs is initiated upon their binding to type I and II transmembrane receptors, which are serine/threonine kinases. The type I receptors phosphorylate Smad1/Smad5/Smad8 form a complex with Smad4. The complex moves into the nucleus and associates there with other DNA-binding proteins, and this large complex recognizes specific DNA motifs, and regulates the transcription of the target genes. This signaling pathway is regulated by a number of factors at multiple steps

the transmembrane receptors (Figure 2). Two types of the transmembrane serine/threonine kinase receptors, types I and II, are required for the signal transduction. The kinase activity of the ligand-bound type II receptor phosphorylates the GS domain of the type I receptor kinase. Substitution mutations of the type I receptor activated the downstream signal transduction without ligand-binding or phosphorylation by the type II receptor (Wieser, Wrana and Massague, 1995). Overexpression of the constitutively active BMP type I receptors, such as BMPR-IA, BMPR-IB and ALK-2, induced osteoblast differentiation in some types of cells (Akiyama *et al*, 1997; Chen *et al*, 1998; Fujii *et al*, 1999; Aoki *et al*, 2001). In contrast, when kinase domain-truncated BMP type I receptors were overexpressed in progenitor cells, the cells failed to differentiate into osteoblasts even in the presence of BMPs (Namiki *et al*, 1997; Chen *et al*, 1998). BMPR-IA is also involved in adipogenic differentiation of calvaria-derived cells (Chen *et al*, 1998). The binding of BMPs to receptors is regulated at multiple steps (Figure 2). The BMP type II receptors increase the ligand binding affinity of the type I receptors (Rosenzweig *et al*, 1995; Beppu *et al*, 1997). BAMBI, a pseudoreceptor of the TGF-β family, stably associates with type I receptors and inhibits BMP-, TGF-β- and activin- induced signals by preventing the formation of receptor complexes. (Onichtchouk *et al*, 1999). Several secreted proteins such as chordin, noggin, follistatin and DAN family members bind to BMPs and act as antagonists that inhibit the binding of the BMPs to receptors (Piccolo *et al*, 1996, 1999; Zimmerman, De Jesus-Escobar and Harland, 1996; Hsu *et al*, 1998; Iemura *et al*, 1998). Defects in joint development are observed in noggin-deficient mice (Brunet *et al*, 1998). BMP-1 acts as a protease that releases the carboxy-terminal propeptide from type I collagen (Kessler *et al*, 1996). Interestingly, a Xenopus homolog of BMP-1 releases active BMPs from the chordin–BMP complex by cleaving chordin (Piccolo *et al*, 1997).

Role of Smads in osteoblast differentiation

Smad transcription factors are substrates of the activated type I receptor kinases in the cytoplasm. The phosphorylated Smad proteins move into the nucleus, bind to the regulatory regions of target genes, and regulate their transcription. Thus, Smad proteins are key molecules in the transduction of signals from the cell membrane to the nucleus (Sakou, 1998; Miyazono, 1999; Massague, 2000; Massague and Chen, 2000; Wrana, 2000; Shi, 2001). So far nine Smad proteins have been identified in vertebrates. They are classified into three subgroups, R-Smad, Co-Smad and I-Smad, according to their structure and function. The R-Smads consist of Smad1, Smad2, Smad3, Smad5 and Smad8. They are directly phosphorylated by the type I receptors at the carboxy terminal SSXS motif (Kretzschmar *et al*, 1997). BMP type I receptors phosphorylate Smad1, Smad5 and Smad8, while TGF-β and activin type I receptors phosphorylate Smad2 and Smad3.

Overexpression of Smad1, Smad5 or Smad8 induces ALP activity and osteocalcin production in C2C12 and 10T1/2 cells (Yamamoto *et al*, 1997; Nishimura *et al*, 1998; Fujii *et al*, 1999; Kawai *et al*, 2000). Smad4 is one of the Co-Smads, which cooperate with all R-Smads. In contrast, both Smad6 and Smad7 inhibit signal transduction of the TGF-β superfamily members, so they are known as I-Smads (I indicates 'inhibitory'). I-Smads appear to be involved in a negative-feedback loop of the TGF-β superfamily signaling, because the expression of I-Smad mRNAs is rapidly induced by BMPs and TGF-βs. Signals other than Smad-mediated ones are also activated by the BMP type I receptors (Lou *et al*, 2000; Gallea *et al*, 2001). Therefore, Smad signals are regulated positively and negatively not only by Smads but also by transcriptional activators and/or repressors (Figure 2). Recently, Yoshida *et al* (2000) reported that tob-null mice have a greater bone mass, and their orthotopic bone formation is elevated relative to that in normal mice in response to BMP-2. They also showed that tob protein negatively regulates osteoblast proliferation and differentiation by suppressing the activity of R-Smads. BMP-2 and leukemia inhibitory factor synergistically stimulated astrocyte differentiation through the formation of a complex between Smad1 and STAT3, bridged by p300 protein (Nakashima *et al*, 1999). Thus, Smads appear to regulate the target gene expression through interaction with other transcription regulators.

Role of Runx2 and Osterix in osteoblast differentiation

The establishment of cbfa1-null mice clearly indicated that this transcription factor is essential for osteoblast differentiation, because, the mutant mice have no bone tissue or osteoblasts (Komori *et al*, 1997; Otto *et al*, 1997). Cbfa1/pebp2aA/AML3/osf-2 is a mammalian homolog of the Drosophila runt, and is now called Runx2. Moreover, Runx2 has also been identified as a gene responsible for cleidocranial dysplasia (CCD), an autosomal-dominant disease with abnormalities in bones formed by intramembranous ossification (Lee *et al*, 1997; Mundlos *et al*, 1997). The null mutation of Runx2 severely affects osteoblast differentiation but causes no abnormality in the patterning of the skeleton (Komori *et al*, 1997; Otto *et al*, 1997). Osteoblasts express high levels of Runx2 *in vivo* and *in vitro*. Runx2-deficient mice lack hypertrophic chondrocytes, suggesting that Runx2 also regulates chondrocyte differentiation (Komori *et al*, 1997). However, recent studies have revealed the complex role of Runx2 in osteoblast and chondrocyte differentiation. Overexpression of Runx2 in some non-osteoblastic cells induced the expression of osteoblast-related genes (Ducy *et al*, 1997; Harada *et al*, 1999). In contrast, Runx2 overexpression in osteoblasts *in vitro* suppressed the expression of type I collagen (Tsuji, Ito and Noda, 1998). Transgenic mice overexpressing either a dominant-negative or wild-type form of Runx2 in osteoblasts exhibited osteopenia (Ducy *et al*, 1999; Liu *et al*, 2001). Runx2 overexpression in chondrocytes *in vivo* caused acceleration of endochondral ossification in mice because of precocious chondrocyte maturation (Takeda *et al*, 2001; Ueta *et al*, 2001). In contrast, overexpression of dominant-negative Runx2 in chondrocytes *in vivo* suppressed their maturation and delayed endochondral ossification (Ueta *et al*, 2001). Furthermore, continuous expression of wild-type Runx2 in non-hypertrophic chondrocytes partially induced mineralization of cartilage in Runx2-null mice (Takeda *et al*, 2001). However, transdifferentiation from chondrocytes into osteoblasts was not observed in these mice (Takeda *et al*, 2001). Thus, Runx2 plays intricate roles in osteoblast and chondrocyte development.

Bone morphogenetic proteins up-regulate Runx2 mRNA expression *in vitro* (Ducy *et al*, 1997; Tsuji *et al*, 1998). Hanai *et al* (1999) showed that R-Smads interact with Runx1/Runx2/Runx3. Zhang, Yasui and Ito (2000) also reported that a truncated Runx2 identified in a CCD patient failed to interact with Smads. Runx2 cooperated with Smads to induce osteoblast differentiation of C2C12 cells (Lee *et al*, 2000; Zhang *et al*, 2000). These lines of evidence suggest that Runx2 interacts tightly with BMP signaling through Smads in osteoblast differentiation. Further studies will be necessary to reveal the precise relationship between Runx2 and transcription factors, including Smads, in the induction of osteoblast differentiation. Elucidation of the regulatory mechanism of osteoblast differentiation will provide a new approach to the treatment of oral diseases.

Recently, Nakashima *et al* (2002) identified a novel zinc finger-containing transcription factor, named Osterix, from C2C12 cells treated with BMP-2. In Osterix-null mice, no bone formation occurred although Runx2 was expressed. Interestingly, however, Osterix was not expressed in Runx2-null mice. These results suggest that Osterix acts downstream of Runx2 during bone development.

Regulation of osteoclast differentiation

Osteoblasts regulate osteoclastogenesis

Development of osteoclasts proceeds within the local microenvironment of bone. A coculture system of mouse osteoblasts/stromal cells and hemopoietic cells was developed to investigate the regulatory mechanisms of osteoclast differentiation (Takahashi *et al*, 1988; Suda, Takahashi and Martin, 1992). Osteoclast-like multinucleated cells are formed in the cocultures in response to various osteotropic factors including 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], PTH, prostaglandin E_2 (PGE_2) and interleukin 11 (IL-11). Those multinucleated cells formed in the coculture expressed major characteristics of osteoclasts such as tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts), calcitonin receptors, $p60^{c\text{-}src}$, vitronectin receptors ($\alpha v\beta 3$), and the ability to form resorption pits on bone and dentine slices. Some mouse stromal cell lines such as MC3T3-G2/PA6 and ST2 resemble calvarial osteoblasts and support osteoclastogenesis in cocultures with mouse spleen cells (Udagawa *et al*, 1989). Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is required to induce osteoclastogenesis. The target cells of osteotropic factors for inducing osteoclast

formation in the cocultures are osteoblasts/stromal cells (Udagawa *et al*, 1995; Liu *et al*, 1998; Takeda *et al*, 1999; Sakuma *et al*, 2000). Therefore, we have proposed that osteoblasts/stromal cells induce osteoclast differentiation factor (ODF) as a membrane-associated cytokine in response to various osteotropic factors (Suda *et al*, 1992). Osteoclast progenitors recognize ODF through cell-to-cell interaction with osteoblasts/stromal cells and differentiate into osteoclasts.

A method for obtaining highly purified osteoclasts from cocultures was established to investigate the role of osteoblasts/stromal cells in osteoclast function (Jimi *et al*, 1996; Suda *et al*, 1997). Purified osteoclasts cultured on dentine slices failed to form resorption pits. The resorptive capability of the purified osteoclasts was restored when osteoblasts/stromal cells were added to the purified osteoclasts. Cell-to-cell contact between osteoblasts/stromal cells and osteoclasts was required for inducing the pit-forming activity of osteoclasts (Jimi *et al*, 1996). Thus, osteoblasts/stromal cells play essential roles in inducing osteoclast function.

Characteristics of osteoclast progenitors

Several lines of evidence indicate that osteoclast progenitors are hemopoietic cells of the monocyte/macrophage lineage. Osteopetrotic op/op mice cannot produce functionally active macrophage colony-stimulating factor (M-CSF, also called CSF-1) because of an insertion of an extra thymidine in the coding region of the M-CSF gene (Yoshida *et al*, 1990). Experiments using the op/op mouse model have established that M-CSF produced by osteoblasts/stromal cells is a crucial factor for osteoclast formation. Administration of M-CSF to op/op mice restored impaired bone resorption (Felix, Cecchini and Fleisch, 1990; Kodama *et al*, 1991). Osteoclast progenitors in the spleen obtained from op/op mice differentiated into osteoclasts in cocultures with normal osteoblasts (Takahashi *et al*, 1991). However, calvarial osteoblasts prepared from op/op mice failed to support osteoclast development in cocultures with normal spleen cells, and the addition of M-CSF to the cocultures induced osteoclast formation in response to $1,25(OH)_2D_3$. These findings indicate that M-CSF produced by osteoblasts/stromal cells plays an essential role in osteoclast development. Mouse peripheral blood mononuclear cells and alveolar macrophages differentiated into osteoclasts in coculture with ST2 cells, a supportive stromal cell line (Udagawa *et al*, 1990). The results of disruption of the PU.1 gene in mice also supported the monocyte/macrophage origin of osteoclasts (Tondravi *et al*, 1997). PU.1 is a myeloid- and B-cell-specific transcription factor, and PU.1(–/–) mice were found to be osteopetrotic. The development of both osteoclasts and macrophages was arrested in PU.1(–/–) mice, suggesting that this transcription factor regulates the initial stage of myeloid differentiation.

Discovery of new TNF receptor-ligand family members involved in osteoclastogenesis

The recent discovery of new members of the TNF receptor-ligand family has clarified the precise mechanism by which osteoblasts/stromal cells regulate osteoclast differentiation and function. Simonet *et al* (1997) cloned a new member of the tumor necrosis factor (TNF) receptor family, termed osteoprotegerin (OPG), in an expressed sequence tag cDNA project. OPG lacks a transmembrane domain and represents a secreted TNF receptor member. Hepatic expression of OPG in transgenic mice results in osteopetrosis. Tsuda *et al* (1997) independently isolated a novel protein termed osteoclastogenesis inhibitory factor (OCIF) from the conditioned medium of human fibroblast cultures. The sequence of the cDNA for OCIF was identical to that of the cDNA for OPG. OPG strongly inhibited osteoclast formation induced by $1,25(OH)_2D_3$, PTH, PGE_2 or IL-11 in cocultures. Using OPG as a probe, a cDNA with an open reading frame encoding 316 amino acid residues was cloned from an expression library of ST2 cells (Yasuda *et al*, 1998). The OPG-binding molecule was a type II transmembrane protein of the TNF ligand family, and its expression in osteoblasts/stromal cells was up-regulated by osteotropic factors including $1,25(OH)_2D_3$, PGE_2, PTH and IL-11. A soluble form of this OPG-binding molecule together with M-CSF induced osteoclast formation from spleen cells in the absence of osteoblasts/stromal cells, and this osteoclast formation was completely inhibited by adding OPG. Thus, the OPG-binding molecule satisfied the major criteria of ODF, and therefore this molecule was renamed ODF (Yasuda *et al*, 1998). Lacey *et al* (1998) also cloned a ligand for OPG (OPGL), and it was found that OPGL was identical to ODF. Molecular cloning of ODF/OPGL demonstrated that it is identical to TRANCE (TNF-related activation-induced cytokine) (Wong *et al*, 1997) and receptor activator of nuclear factor κB ligand (RANKL) (Anderson *et al*, 1997), which had been independently identified by other research groups. TRANCE was cloned during a search for apoptosis-regulatory genes in mouse T cell hybridomas. TRANCE induced activation of c-Jun N-terminal kinase (JNK) in T lymphocytes and inhibited apoptosis of mouse and human dendritic cells (Wong *et al*, 1997). A new member of the TNF receptor family, termed 'RANK', was cloned from a cDNA library of human dendritic cells (Anderson *et al*, 1997). The mouse homolog was also isolated from a fetal mouse liver cDNA library. The mouse RANK cDNA encodes a type I transmembrane protein of 625 amino acid residues. Thus, ODF, OPGL, TRANCE and RANKL are different names for the same molecule, a protein which is important for the development and function of T cells, dendritic cells and osteoclasts. RANK is the transmembrane signaling receptor for ODF/OPGL/TRANCE/RANKL. OPG/OCIF is a soluble decoy receptor for ODF/OPGL/TRANCE/RANKL. The terms 'RANKL', 'RANK' and 'OPG' are used in this article in accordance with the guidelines of The American Society for Bone and Mineral Research President's Committee on Nomenclature (2000). RANKL stimulates the pit-forming activity of mature osteoclasts (Burgess *et al*, 1999; Jimi *et al*, 1999a). Human osteoclasts are also formed in cultures of human peripheral

blood mononuclear cells in the presence of RANKL and human M-CSF (Matsuzaki *et al*, 1998).

RANKL–RANK interaction in osteoclastogenesis

The expression of RANKL in osteoblasts/stromal cells is up-regulated by osteotropic hormones and factors such as $1,25(OH)_2D_3$, PTH, PGE_2 and IL-11. Compounds that elevate intracellular calcium, such as ionomycin, cyclopiazonic acid and thapsigargin, also induced osteoclast formation in mouse cocultures of bone marrow cells and primary osteoblasts (Takami *et al*, 1997) (Figure 3). Similarly, high calcium concentrations in the culture medium induced osteoclast formation in the cocultures. Treatment of primary osteoblasts with these compounds or the medium containing high levels of calcium stimulated the expression RANKL and OPG mRNAs (Takami *et al*, 2000). These results suggest that independent signals mediated by vitamin D receptors (VDR), cAMP, gp130 and intracellular calcium induce expression of RANKL in osteoblasts/stromal cells (Figure 3).

Receptor activator of NF-κB ligand knockout(–/–) mice exhibit typical osteopetrosis, with total occlusion of the bone marrow space within endosteal bone (Kong *et al*, 1999). RANKL(–/–) mice lack osteoclasts but have normal osteoclast progenitors that can differentiate into functionally active osteoclasts when cocultured with normal osteoblasts/stromal cells. Like RANKL-deficient mice, RANK(–/–) mice are characterized by severe osteopetrosis (Dougall *et al*, 1999). The osteopetrosis observed in RANK(–/–) mice but not RANKL(–/–) mice is rescued by transplantation of normal bone marrow cells, indicating that RANK(–/–) mice have an intrinsic defect in osteoclast lineage cells. These data indicate that RANK is the intrinsic cell surface determinant that mediates the effects of RANKL on bone resorption. A gene mapping study showed that the gene responsible for familial expansile osteolysis and familial Paget's disease of bone mapped to the gene encoding RANK (Hughes *et al*, 2000). This finding confirms that RANK is involved in osteoclast differentiation and activation in humans as well.

Activation of NF-κB and JNK through the RANK-mediated signaling system appears to be involved in the differentiation and activation of osteoclasts. The cytoplasmic tail of RANK interacts with TNF-associated factor 1 (TRAF1), TRAF2, TRAF3, TRAF5 and TRAF6 (Darnay *et al*, 1998; Galibert *et al*, 1998; Wong *et al*, 1998; Darnay *et al*, 1999; Kim *et al*, 1999). TRAF6-mediated signals appear to be important for osteoclast differentiation and function, because TRAF6(–/–) mice develop osteopetrosis with defects in bone resorption and tooth eruption (Lomaga *et al*, 1999; Naito *et al*, 1999). Mice deficient in both the p50 and p52 subunits of NF-κB develop severe osteopetrosis (Franzoso *et al*, 1997; Iotsova *et al*, 1997). The osteopetrotic phenotype was rescued by bone marrow transplantation, indicating that the osteoclast progenitors are inactive in the double-knockout mice. RANKL-induced activation of NF-κB in osteoclast progenitors seems to play a crucial role in osteoclast differentiation. Mice lacking c-Fos also develop osteopetrosis because of an early block of differentiation in the osteoclast lineage (Wang *et al*, 1992; Grigoriadis *et al*, 1994). The dimeric transcription factor activator protein-1 (AP-1) is composed of mainly Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) and Jun proteins (c-Jun, JunB and JunD). These results suggest that AP-1 appears to act downstream of RANK-mediated signals.

Role of inflammatory cytokines in osteoclastogenesis

Since the discovery of the RANKL–RANK signaling system, RANKL has been regarded as the sole factor responsible for inducing osteoclast differentiation. However, recent findings indicate that inflammatory cytokines and LPS are directly involved in osteoclast differentiation and function (Figure 4).

Interleukin-1 directly stimulates osteoclast function through the IL-1 type 1 receptor expressed by osteoclasts (Jimi *et al*, 1999b). The pit-forming activity of

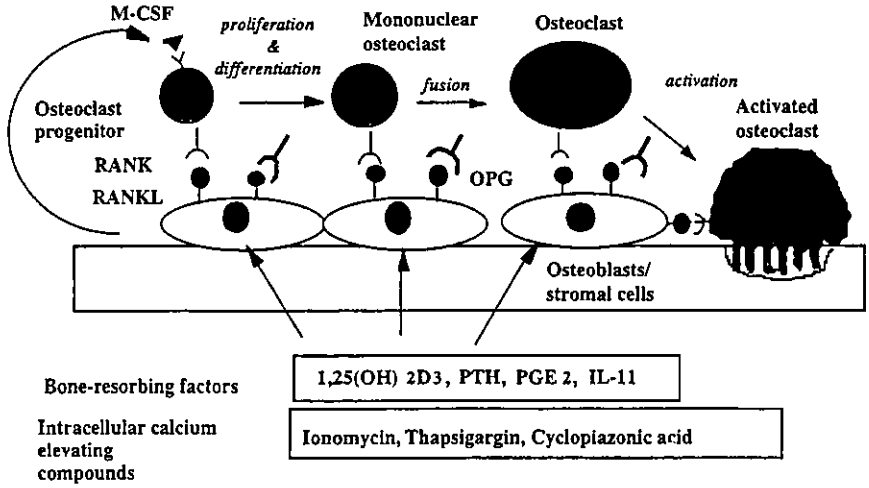

Figure 3 A schematic representation of osteoclast differentiation and function supported by osteoblasts/stromal cells. Osteotropic factors such as $1,25\ (OH)_2D_3$, PTH, PGE_2 and IL-11 stimulate the expression of RANKL as a membrane associated factor in osteoblasts/stromal cells. Compounds that elevate intracellular calcium, such as ionomycin, cyclopiazonic acid and thapsigargin, also induce RANKL expression in osteoblasts/stromal cells. Osteoclast progenitors of the monocyte-macrophage lineage recognize RANKL expressed by osteoblasts/stromal cells through cell-to-cell interaction, and differentiate into osteoclasts. M-CSF produced by osteoblasts/stromal cells is another essential factor for osteoclast differentiation. RANKL expressed by osteoblasts/stromal cells also stimulates osteoclast function through cell-to-cell interaction

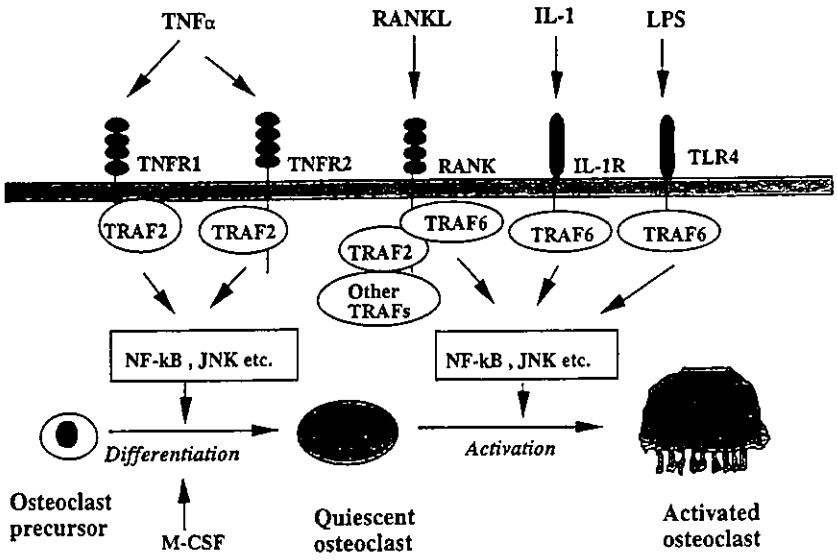

Figure 4 Schematic representation of ligand-receptor systems in osteoclast differentiation and function regulated by TNFα, RANKL, IL-1 and LPS. TNFα and RANKL independently stimulate osteoclast differentiation via TNFR1 and TNFR2, and RANK expressed by osteoclast precursors, respectively. M-CSF is a common factor required for TNFα- and RANKL-induced osteoclast differentiation. TRAF2 and other TRAFs may transduce signals for the differentiation of osteoclasts. The activation of osteoclasts is induced by RANKL, IL-1 and LPS through RANK, type 1 IL-1 receptor and TLR4, respectively. TRAF6 appears to act as a common signal transducer in osteoclast activation induced by RANK, IL-1 and LPS. Signal transduction cascades such as NF-κB and JNK activation may be involved in the differentiation and activation of osteoclasts

osteoclasts induced by IL-1 was completely inhibited by adding IL-1 receptor antagonist (IL-1ra) but not by OPG. LPS is a cell component of Gram-negative bacteria that causes inflammatory bone loss. Recent studies identified mouse toll-like receptor 4 (TLR4) as the receptor for LPS (Poltorak *et al*, 1998; Hoshino *et al*, 1999; Qureshi *et al*, 1999). The cytoplasmic signaling cascade of TLR4 is similar to that of IL-1 receptors. Both receptors have been shown to use TRAF6 as a common signaling molecule. To examine the effect of LPS on the survival and fusion of osteoclasts, mononuclear osteoclasts (preosteoclasts, pOCs) were collected from a mouse coculture system and cultured in the presence or absence of LPS (Suda *et al*, 2001). Most pOCs died within 24 h in the absence of any stimulus. LPS as well as RANKL supported the survival of pOCs, and induced their fusion to form multinucleated osteoclasts. LPS-induced osteoclast formation in pOC cultures was observed even in the presence of OPG and IL-1 receptor antagonists. LPS induced pit-forming activity of pOCs in the presence of M-CSF. These findings suggest that LPS as well as IL-1 stimulates the survival and fusion of pOCs.

Recent studies have shown that TNFα directly stimulates the differentiation of osteoclast progenitors into osteoclasts in the presence of M-CSF (Azuma *et al*, 2000; Kobayashi *et al*, 2000). When mouse bone marrow cells were cultured with M-CSF, M-CSF-dependent bone marrow macrophages appeared within 3 days. Not only RANKL but also TNFα stimulates the differentiation of these macrophages into osteoclasts in the presence M-CSF. Osteoclast formation induced by TNFα was inhibited by the addition of the respective antibodies against TNF receptor type I (TNFRI, p55) and TNF receptor type II (TNFRII, p75), but not by the addition of OPG. These results demonstrate that TNFα stimulates osteoclast differentiation through a mechanism independent of the RANKL–RANK system. It was also reported that when osteotropic factors such as $1,25(OH)_2D_3$, PTHrP and IL-1 were administered to RANK(−/−) mice, neither TRAP-positive cell formation nor hypercalcemia was induced (Li *et al*, 2000). In contrast, administration of TNFα to RANK(−/−) mice induced TRAP-positive cells near the site of injection, although the number of TRAP-positive cells induced by TNFα was not large. This suggests that TNFα induces osteoclast differentiation in the absence of RANK-mediated signals *in vivo*. Lam *et al* (2000) also reported that a small amount of RANKL strongly enhanced osteoclast differentiation in a pure population of murine precursors in the presence of TNFα. These results suggest that RANKL-induced signals cross-communicate with TNFα-induced ones in the target cells (Figure 4). Thus, these cytokines and LPS play important roles in osteoclastic bone resorption induced by inflammatory diseases including periodontitis. Further studies will be necessary to elucidate the regulatory mechanisms of osteoclastic bone resorption induced by inflammatory cytokines and LPS.

Role of TGF-β super family members and interferon-γ in osteoclastogenesis

Bone is a major storage site for TGF-β super family members such as TGF-β and BMPs, and osteoclastic bone resorption releases these cytokines. TGF-β has been shown to enhance osteoclast differentiation in hematopoietic cells stimulated with RANKL and M-CSF (Sells Galvin *et al*, 1999; Quinn *et al*, 2001). Fuller, Bayley and Chambers (2000a) reported that activin A also powerfully synergized with RANKL for induction of osteoclasts from their progenitors. Moreover, osteoclast formation induced by RANKL was completely abolished by soluble activin receptor type IIA or soluble TGF-β receptor II, suggesting that activin A and TGF-β are essential factors for osteoclastogenesis (Fuller *et al*, 2000a; b). We also showed that BMP-2 strikingly stimulated osteoclast differentiation in the presence of RANKL and M-CSF (Itoh *et al*, 2001). OPG completely inhibited osteoclast differentiation induced by RANKL and BMP-2. A soluble form of

BMP receptor type-IA also inhibited osteoclast formation in the presence of RANKL (Itoh *et al*, 2001). We found that BMP receptor type IA mRNA was expressed on not only osteoclast progenitors but also mature osteoclasts, and that BMP-2 enhanced the survival of purified osteoclasts in the presence of RANKL but not M-CSF (Itoh *et al*, 2001). Smad1 and Smad5 are involved in the BMP signals, whereas Smad2 and Smad3 in the TGF-β signals in the target cells. However, both BMP and TGF-β showed similar effects on osteoclast progenitors. This suggests that signaling pathways other than Smad-mediated pathways are involved in enhancement of RANKL-induced osteoclast differentiation by TGF-β super family members. Further studies are necessary to elucidate the molecular mechanism of the crosstalk between BMPs and RANKL in osteoclastogenesis.

Bone resorption is regulated by the immune system, where T-cell expression of RANKL may contribute to pathological conditions, such as periodontitis and autoimmune arthritis. Activated T cells also produce interferon (IFN)-γ, which strongly suppresses osteoclastogenesis by interfering with the RANKL–RANK signaling pathway. Takayanagi *et al* (2000) reported that IFN-γ induced rapid degradation of TRAF6, which resulted in strong inhibition of the RANKL-induced activation of NF-κB and JNK. This inhibition of osteoclastogenesis was rescued by overexpressing TRAF6 in precursor cells, suggesting that TRAF6 is the target critical for the IFN-γ action. These results indicate that there is crosstalk between the TNF and IFN families of cytokines, through which IFN-γ provides a negative link between T-cell activation and bone resorption.

Conclusion

Bone morphogenetic proteins play critical roles in osteoblast differentiation. Smad-mediated signals are essential in BMP-induced osteoblast differentiation. Runx2 and Osterix are transcription factors required for osteoblast differentiation and bone formation. RANKL–RANK interaction is absolutely necessary for osteoclast differentiation. LPS and some inflammatory cytokines such as TNFα and IL-1 are directly involved in osteoclast differentiation and function through a mechanism independent of RANKL–RANK interaction. TGF-β super family members and IFN-γ are also important regulators in osteoclastogenesis. Further studies on the regulatory mechanisms of osteoblast and osteoclast differentiation will provide novel approaches for the treatment of bone and oral diseases.

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