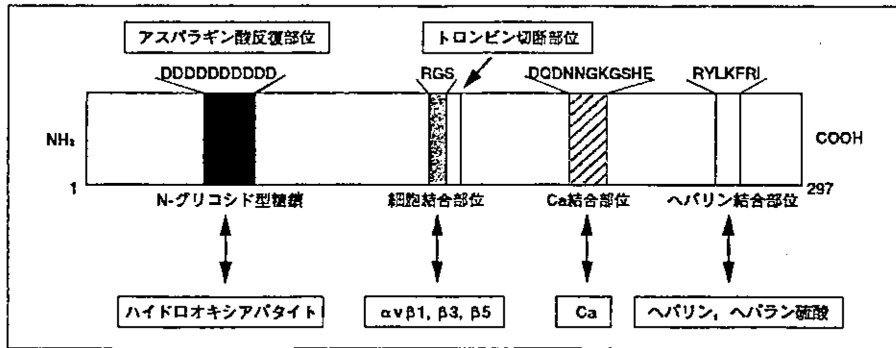

図1　オステオポンチンの構造

I. OPNの構造（図1）

OPNは約300個のアミノ酸からなる，分子量約32,000のポリペプチドを骨格にもつ分泌型リン酸化糖タンパク質分子である。リン酸化や糖付加の程度により分子量は44,000～75,000に変化する。OPNはグルタミン，グルタミン酸，アスパラギン，アスパラギン酸が総アミノ酸の半数以上を占める特徴的なタンパク分子である。また，中央部にはトロンビン切断部位が存在し，そのすぐN末端側には細胞接着ドメインと考えられるRGD配列をもつ。さらに，カルシウムやヒドロキシアパタイトなどに親和性の高いドメインをもち，骨などの石灰化基質に高い親和性をもつ。

II. 破骨細胞による骨吸収におけるOPNの役割

最近，主にノックアウトマウスを用いた研究により，破骨細胞による骨吸収の分子機構が明らかとなってきた[42]。破骨細胞による骨吸収の最終ステップでは，破骨細胞は骨へ強固に接着する必要があるが，この破骨細胞と骨の接着にはOPNと$\alpha v\beta 3$インテグリンとの結合が必須と考えられている。実際に，$\alpha v\beta 3$インテグリンに対する抗体でこの結合を阻害すると破骨細胞による骨吸収は抑制される[43]。また，OPNノックアウトマウスにおいては軽度の大理石病が観察され，$\beta 3$インテグリンノックアウトマウスでも同様に軽度の大理石病が観察される。これらの事実から，OPNはインテグリンとの結合により破骨細胞による骨吸収において促進的な役割を果たしているものと考えられる。

III. 骨吸収性疾患

骨は，絶えず骨形成と骨吸収を繰り返しターンオーバーされ，維持されている。したがって，正常な（健康な）骨を維持するためには骨形成と骨吸収のバランスが保たれなければならない。そのバランスが破綻し骨吸収へ傾くと，病的な骨，つまり骨吸収性疾患を生じることになる。骨吸収性疾患は，病態病理学的に①非炎症性のもの，②炎症性のもの，③腫

表1　オステオポンチンの多彩な生物学的作用と病態・疾患との関わり（文献4～41より）

細胞・組織	生物学的作用と病態・疾患との関わり	文献
骨	ミネラルの骨への沈着を制御	4,5
	荷重シグナルの伝達	6
	破骨細胞の骨への接着	7,8
	骨吸収性疾患（骨粗鬆症，関節リウマチ，多発性骨髄腫）	9-15
免疫系	TH1免疫の初期誘導	16
	IL-12，IFNγの産生亢進，IL-10の産生制御	
	単球，マクロファージの遊走	17,18
	B細胞の抗体産生を促進	19,20
	TH1疾患（関節リウマチ，多発性硬化症）	21
心・血管系	再狭窄	22,23
	粥状動脈硬化のプラーク形成，石灰化	24,25
	心筋症	26
腎臓・尿路系	NO産生の抑制	27
	尿路結石の形成の阻止	28
	腎炎	29-31
脳	虚血（脳卒中）	32
肉芽腫形成	創傷，結核，サルコイドーシス	33-36
腫瘍	細胞のトランスフォーメーション，癌	37-41

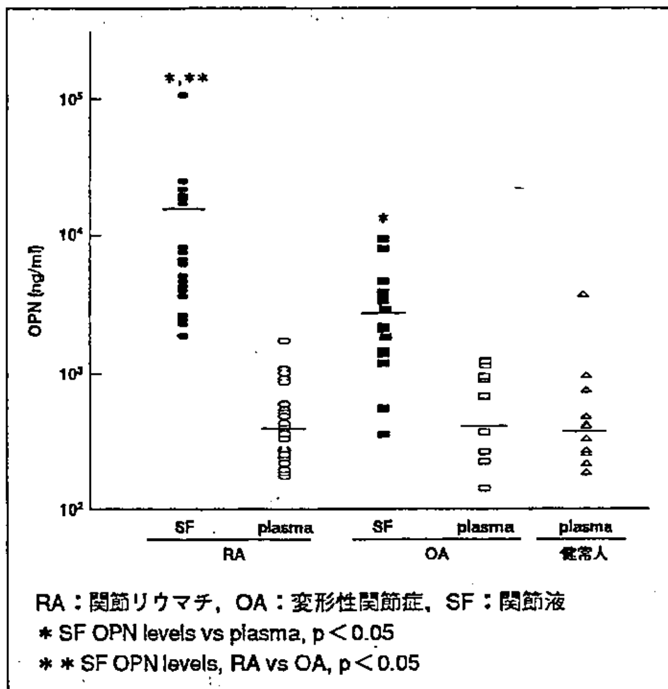

図4　血中・関節液中のOPNレベル（文献12より）

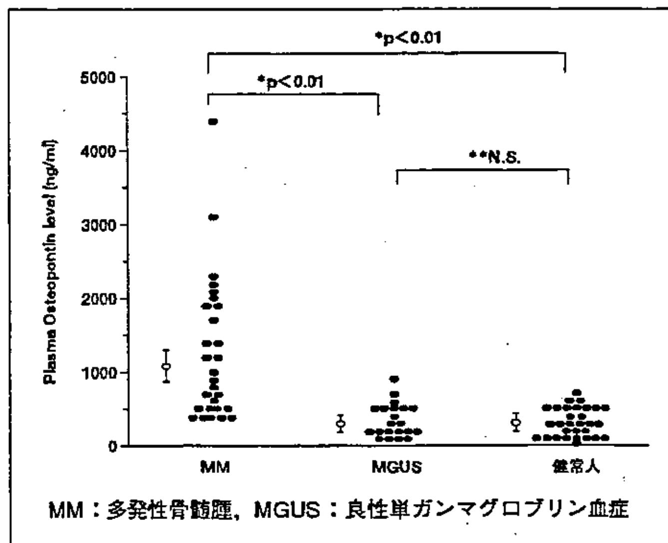

図5　血中OPNレベル（文献15より）

ていることが示唆されている。実際に，炎症性関節炎の代表的疾患であるRAにおいても滑膜組織でのOPNの発現は亢進し，RAの関節液中には健常人の血中レベルの約10～1000倍のOPNが存在する(図4)[12]。このことから，RAの関節局所では，大量のOPNが産生され，骨破壊を促進していることが考えられる。さらに，この関節液中のOPNレベルの上昇はCRPなどの炎症のマーカーと強く相関しており，OPNの産生亢進がRAの疾患活動性と関連していることが示唆されている[12]。また，*in vitro*においてOPNが軟骨細胞に作用し，MMP-1（matrix metalloproteinase-1, collagenase-1）の産生を誘導することも報告されている[13]。さらに，RAの血中ではトロンビンで分断されたOPNが増加しており[12]，最近，トロンビンで分断された後に表出するOPNの潜在的エピトープの重要性も指摘されている[14]。

3．腫瘍性骨吸収性疾患とOPN

腫瘍性骨吸収性疾患の代表的なものとして多発性骨髄腫（multiple myeloma：MM）がある。MMは形質細胞の悪性腫瘍であるが，全身性の骨吸収性骨破壊を1つの特徴とし，このために強い骨痛や病的骨折などが臨床上大きな問題となることが多い。MMの骨吸収部位には他の骨吸収性疾患の場合と同様に活性化された破骨細胞が観察されることから，MMの骨吸収も主に破骨細胞による骨吸収の亢進が原因と考えられている。そして，以前から骨髄腫細胞から破骨細胞活性化因子（osteoclast activating factor：OAF）が産生されているものと推察されている。OAFについては，IL-1，TNFβ，IL-6などの炎症性サイトカインが候補として考えられていたが，まだ直接的な責任分子は同定されていない。最近，われわれはMM患者の血清中のOPNレベルが，健常人やMGUS（monoclonal gammopathy of undetermined significance, MMの前臨床状態の良性単ガンマグロブリン血症）患者に比べ有意に上昇していることを見出した（図5）[15]。また，このMM患者の血中のOPNレベルは，MMの臨床病期や骨病変の有無と相関していた[15]。さらに，MM患者の骨髄細胞やMM患者由来のcell lineは大量のOPNを産生していた[16]。このことから，腫瘍性骨吸収性疾患のMMにおける骨破壊の原因分子としてのOPNの関与が示

唆される。

おわりに

OPNは組織のリモデリングに関わる多彩な作用をもつサイトカインであり，種々の疾患において注目されている。特に，OPNは閉経後の骨粗鬆症，関節リウマチ，多発性骨髄腫など種々の骨吸収性疾患において骨破壊因子として作用していることが強く示唆され，これらの疾患の重要な診断や活動性の指標，および治療上の標的分子となることが考えられる。

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II. 骨代謝調節系

破骨細胞の機能・骨吸収メカニズム

概論：破骨細胞の分化・骨吸収調節機構

Regulatory mechanism of osteoclast differentiation and function

奥村茂樹[1]　宇田川信之[1]　髙橋直之[2]

Key words：破骨細胞，骨芽細胞，骨吸収，RANKL，極性化

はじめに

いったん形成された骨組織は，なぜ破骨細胞による吸収と骨芽細胞による形成を繰り返すのだろうか．一定の形態を保つかに見える骨も，骨吸収と骨形成を絶えず繰り返す動的な組織である．破骨細胞と骨芽細胞の機能バランスが崩れると，骨粗鬆症や大理石骨病などの骨疾患が引き起こされる．骨には少なくとも2つの役割がある．1つは力学的に身体を支える‘支柱’としての役割であり，もう1つはカルシウム(Ca)貯蔵庫としての役割である．骨の支柱的役割から骨疾患を考えると，破骨細胞は文字どおり‘骨を破壊する細胞’である．しかし，骨をCaの貯蔵庫として考えると，破骨細胞は血中にCaを動員する‘Ca濃度調節細胞’といえる．骨密度の低下も，血中のCa濃度の異常もともに危険である．

本稿では，この骨吸収を担う破骨細胞が，どのように形成され骨吸収能を示すのか，概略的ではあるが順を追って述べたい．

1. 破骨細胞としての形質獲得

破骨細胞は骨組織にのみ存在し，骨吸収を担う多核細胞である．単球/マクロファージ系の破骨細胞前駆細胞が骨組織において単核の破骨細胞に分化した後，互いに融合し，多核の破骨細胞が形成される[1]．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により厳密に調節されている．1998年，骨芽細胞に発現し，破骨細胞の分化と機能を調節する破骨細胞分化因子RANKL(receptor activator of NF-κB ligand)がクローニングされ[1]，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(図1)．すなわち，骨芽細胞は破骨細胞の分化に必須な因子であるM-CSF(macrophage colony-stimulating factor)とRANKLを発現する[1]．一方，破骨細胞前駆細胞は，M-CSF受容体(c-fms)とRANKL受容体(RANK)を発現する．破骨細胞前駆細胞は，骨芽細胞との細胞間接触を介してRANKLを認識し，M-CSFの存在下で破骨細胞に分化する．また，骨芽細胞は，RANKLのデコイレセプターであるOPG(osteoprotegerin)も分泌する．OPGは，RANKLとRANKの結合を競合的に阻害する骨吸収抑制因子である．

骨芽細胞によるM-CSFの発現は恒常的であるのに対し，RANKLの発現は1,25(OH)$_2$D$_3$(1,25-dihydroxyvitamin D$_3$)，PTH(parathyroid hormone)，PGE$_2$(prostaglandin E$_2$)，IL-11(interleukin 11)などの骨吸収因子により誘導される[1]．1,25(OH)$_2$D$_3$はビタミンD受容体(VDR)

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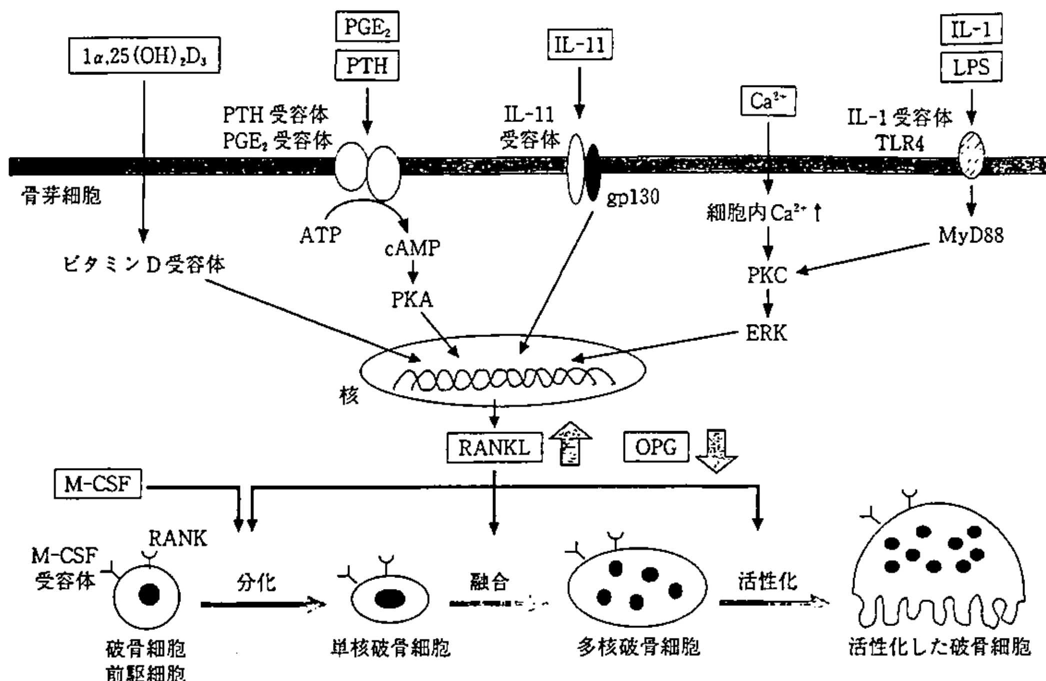

図1 骨芽細胞におけるRANKLの発現誘導とRANKLの作用

を介し，IL-11やIL-6は双方に共通のシグナル伝達因子であるgp130を介してRANKLの発現を上昇させる．また，PTHやPGE_2の受容体からのシグナルは，cAMP/PKAを介してRANKL発現を上昇させると考えられる．これらの骨吸収因子は，骨芽細胞によるOPGの産生も抑制し，破骨細胞形成を促進する(図1)．更に，骨芽細胞の細胞内Caレベルが上昇するような薬物や培養液のCa^{2+}濃度の増加はRANKLの発現を誘導する[2]．この細胞内Caレベルの上昇を介するRANKLの発現誘導は，PKC(protein kinase C)とERK(extracellular signal-regulated kinase)が仲介するという結果を著者らは得ている．IL-1とLPS(リポ多糖)は骨芽細胞のRANKLの発現を誘導するが，この発現誘導はPKC/ERKを介する．一方，分化した成熟破骨細胞もRANKを発現しており，RANKLからの刺激により骨吸収活性が誘導される[1]．

単球/マクロファージ系細胞は破骨細胞への分化に伴い，貪食能や抗原提示能など単球/マクロファージ特有の形質を失い，骨吸収能を獲得する．破骨細胞のマーカー分子としてカテプシンKやカルシトニン受容体，$p60^{c-src}$，酒石酸抵抗性酸ホスファターゼ(TRAP)などが知られている．単球/マクロファージ系前駆細胞から分化した単核破骨細胞は，骨吸収能をはじめとするこれらのマーカーをすべて発現する[1]．そのため，破骨細胞分化を誘導する重要なシグナルは単核破骨細胞への分化で完結すると考えられる(図1)．

2. 単核破骨細胞の融合

単核破骨細胞は融合して多核細胞を形成する．破骨細胞はなぜ多核化するのか，その生理的意義は不明であるが，正常な骨吸収を行うために重要であると推測される．細胞-細胞間の膜融合は，破骨細胞の形成のみならず，ウイルス感染や受精，筋芽細胞から筋管細胞の形成時にも認められる．エキソサイトーシスやエンドサイトーシスなど，細胞内の膜融合は広く研究されているが，細胞間の膜融合機構はほとんど解明されていない．破骨細胞の研究においても，細

胞間融合機構の研究は大きく遅れている．しかし，幾つか興味深い知見も報告されているので紹介したい．

細胞間融合には，細胞膜表面の糖，コレステロールのような脂質，更に細胞外の酸性環境なども関与していると考えられている[3]．倉地らは，破骨細胞間融合にマンノースが関与する可能性を指摘した[4]．マウスの骨芽細胞と造血細胞の共存培養系において，マンノース残基に結合するpradimicinを培養初期(0-3日)に添加しても多核破骨細胞の形成には影響が認められないが，単核破骨細胞が融合する時期に添加すると，多核化が抑制されたという．更に，破骨細胞前駆細胞の膜表面マンノース量が，分化に伴って増大することを示し，破骨細胞の融合に細胞膜表面のマンノースが重要な役割を果たしている可能性を報告した．

また，細胞融合に関与する膜蛋白として，ADAM(a disintegrin and metalloprotease domain)が注目される．ADAMファミリーメンバーは，metalloprotease domain，disintegrin domain，cysteine-rich domain，EGF様リピートなど，多様なドメインを有する[5]．これらの中で，インテグリンが結合するdisintegrin domainは細胞融合にも関与する可能性が指摘されている．受精に関与するfertilinや筋芽細胞の融合に関与する分子として発見されたmeltrinもADAMファミリーメンバーである．meltrinは3種類のイソフォーム(α, β, γ)がクローニングされている．このうち，αとβは骨格筋と骨にのみ発現しているという[6]．破骨細胞の融合にmeltrin-αが関与する可能性が報告された[7]．興味深いことは，マンノースとmeltrin-αは，ともに融合に必要な時期にのみ発現し，融合が終了すると発現が低下することである．実際の骨表面における細胞間融合過程は不明だが，関連分子の発現が厳密に制御されていると考えられる．

細胞運動も細胞融合を考えるうえで重要である．インテグリンは，RGD配列を認識する細胞外マトリクスの受容体で，接着と細胞運動にかかわる膜蛋白である．破骨細胞は数種類のインテグリンを発現している．とりわけ，$\alpha_v\beta_3$インテグリン(ビトロネクチンレセプター)が強く発現しているため，その役割が注目される．仲村らは，$\alpha_v\beta_3$インテグリンに特異的に結合するechistatinが単核破骨細胞の融合を抑制することを報告した[8]．更に，M-CSFが誘導する単核破骨細胞の運動を阻害するという．このように，$\alpha_v\beta_3$インテグリンは細胞運動を調節し，破骨細胞の細胞融合を促進するものと考えられる．一方，インテグリンが細胞融合に直接関与している可能性も指摘される．マウスの受精では，卵表面の$\alpha_6\beta_1$インテグリンと精子のfertilin-βが結合し，細胞融合が起こる[5]．ADAMファミリーメンバーとインテグリンによる異分子間相互作用は，破骨細胞の融合にも関与しているかもしれない(図2)．

単核破骨細胞の融合を直接促進する因子として，RANKL，M-CSF，IL-1，TNFα，LPSがあげられる．転写因子NFAT2(NFATc1)が破骨細胞の分化を誘導することが報告され，その役割が注目されている[9,10]．Ishidaらは，破骨細胞に分化する初期に上昇する遺伝子としてNFAT2を見いだした[9]．NFAT2の活性化を阻害する薬物サイクロスポリンAは，RANKLが誘導するRAW264細胞の単核破骨細胞の出現は抑制しないが多核破骨細胞の出現を抑制したという．更に，NFAT2のantisenseをRAW264細胞に発現させると，RANKL誘導性のTRAP-陽性単核細胞の出現は抑制されないが，多核化が抑制されることを報告した．最近，NFAT1(NFATc2)は，IL-4の産生の促進を介して筋芽細胞の融合を促進することが報告された[11]．このように，NFATが破骨細胞の融合過程を調節している可能性も示唆される．

3. 活性化による骨吸収能獲得

このようにして多核化した破骨細胞は，どのように骨吸収活性を発現するのだろうか．骨吸収を行っている破骨細胞は，骨表面に明帯(clear zone)と波状縁(ruffled border)を形成し，細胞極性を示す(図3)．破骨細胞は，波状縁よりプロトン(H^+)と塩素イオン(Cl^-)を骨面に放

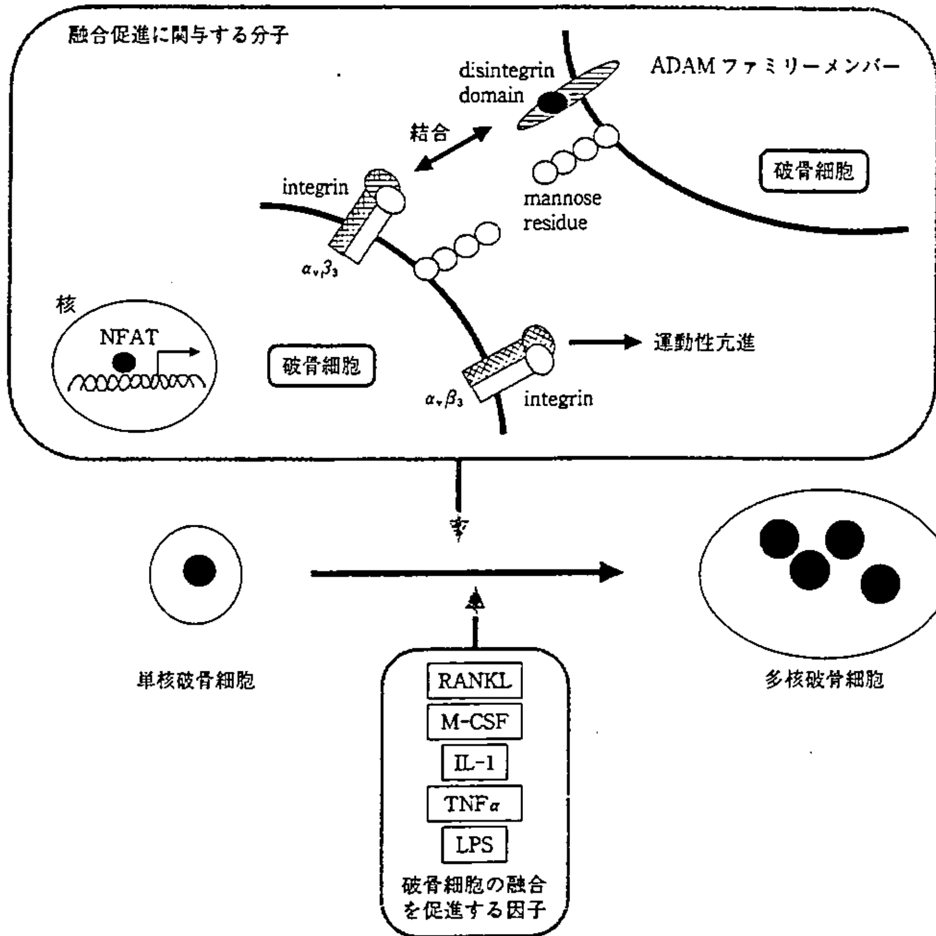

図2 破骨細胞の融合を促進する因子と融合促進に関与する分子

出し，ヒドロキシアパタイト結晶を脱灰する．そのため，波状縁にはV-ATPase(液胞型プロトン-ATPase)とClチャネルが集積している(図4)．また，波状縁からカテプシンKなどの蛋白分解酵素も分泌され，骨基質蛋白の分解も促される．

このような骨吸収を開始するには，少なくとも2つのシグナル系が同時に作動する必要があると考えられる．接着装置ポドソームを介するシグナルとTRAF6(TNF receptor associated factor 6)を介するシグナルである(図3)．破骨細胞は，$\alpha_v\beta_3$インテグリンを介して骨基質に接着する．接着により，Pyk2(prolin-rich tyrosine kinase 2(focal adhesion kinaseのホモログ))，p130[Cas]，p60[c-src]などが接着点にリクルートされ，接着シグナル複合体が形成される[12,13]．骨に接着したというシグナルは，この複合体を介し，波状縁形成誘導シグナルを細胞内に伝達する[12,13]．

しかし，純化した破骨細胞は，骨基質に接着させても骨吸収能を発現しないため，他のシグナルも必要であることがわかる．様々なサイトカインを検索したところ，RANKL，IL-1，LPSが，純化した破骨細胞の骨吸収(波状縁形成)を誘導することが示されている[1,13]．RANK，IL-1受容体，Toll-様受容体4(TLR4，LPS受容体)は，TRAF6を共通のシグナル伝達分子として利用することから，破骨細胞の波状縁形成には

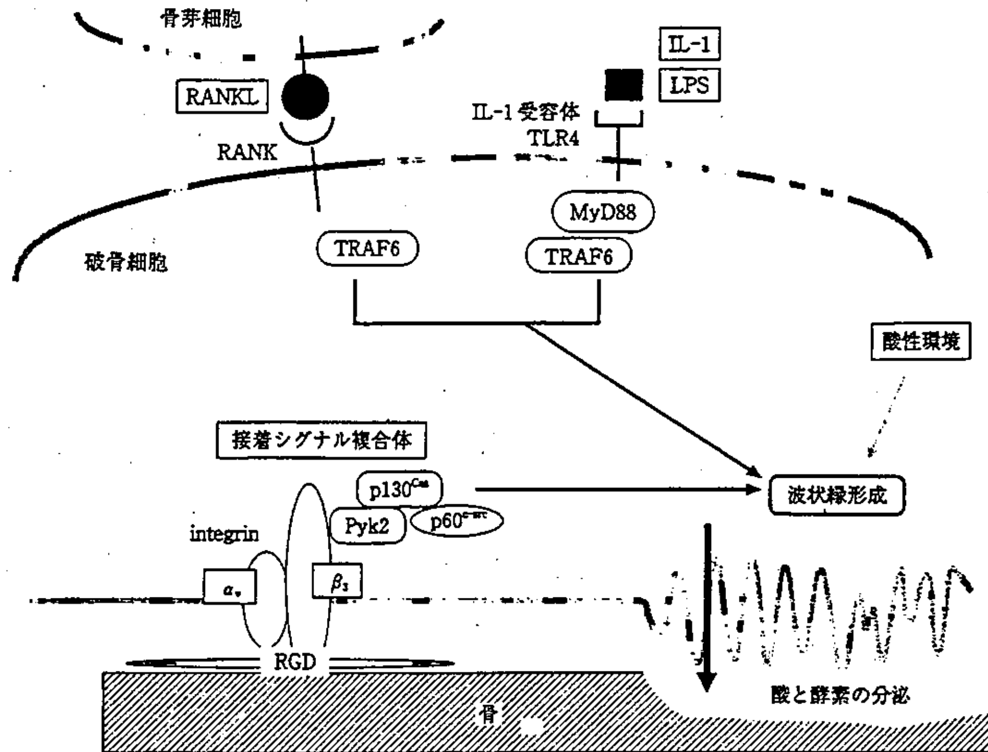

図3 波状縁形成を誘導するシグナル系

TRAF6を介するシグナルも必要と考えられる[1].

更に，骨吸収を促進する要因として，酸性環境があげられる．酸性環境は，破骨細胞のcarbonic anhydrase IIのmRNAの発現[14]やポドソーム形成を促進し[15]，V-ATPaseの活性を亢進させることが報告されている．実際，酸性環境にすると吸収窩形成能が著しく増加する[16]．一方，V-ATPaseはTriton x-100などの界面活性剤による可溶化に抵抗性を示し，不溶性分画に残ることから，細胞骨格によって運ばれる可能性が示唆される[17]．破骨細胞に特異的に認められるV-ATPaseのa3サブユニットに対する特異抗体を用いて免疫染色すると，微小管の染色像と一致する[18]．V-ATPaseの特異的阻害剤であるbafilomycin Aで破骨細胞を処理すると，酸分泌のみでなく波状縁の形成も抑制されるため，V-ATPase活性自体が，破骨細胞の極性化に関与すると考えられる[19]．a3サブユニットに異常をもつ大理石骨病マウス(oc/ocマウス)の破骨細胞では，骨表面側へのV-ATPaseの集積が認められず，波状縁形成も認められない[17]．このように酸分泌と波状縁形成は密接につながっているものと推察される．

4. 極性化した破骨細胞の機能

Väänänenらは，骨吸収を行っている破骨細胞の機能と構造を解析し，興味ある所見を報告した[20,21]．破骨細胞は，消化した蛋白を吸収小胞(transcytotic vesicle)を形成して波状縁領域から取り込み，細胞内移送(トランスサイトーシス)を経てapical領域から分泌しているという．彼らは，このapical領域をfunctional secretary domain(機能的分泌領域：FSD)と名付けている．FSDには，ある種のウイルス蛋白が特異的に輸送されること，また吸収小胞がFSDに集積することから，他の膜領域と異なる膜構造をもつという[20,21]．したがって，骨吸収を行っている破骨細胞の細胞膜は，機能的にも異な

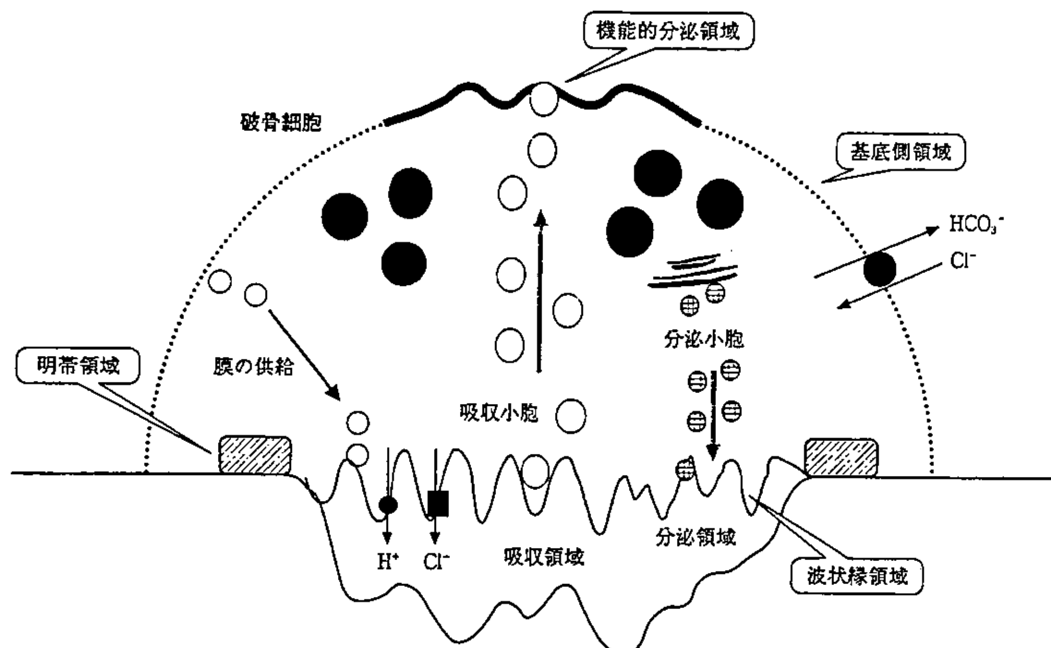

図4 骨吸収を行っている破骨細胞のもつ4つの異なる膜領域とその役割

る4つの膜領域(①波状縁領域(ruffled border domain), ②明帯領域(clear zone domain), ③基底側領域(basolateral domain), ④機能的分泌領域(FSD))に分けられる(図4).

波状縁領域は, 吸収窩に面する部位に形成される複雑に入り組んだ膜ドメインで, 酸や蛋白分解酵素を分泌するとともに消化した蛋白を吸収する. そのため, 波状縁領域は更に分泌領域と吸収領域に分離されるという[21].

明帯領域の実体は, F-アクチンのドット(ポドソーム)がリング状に集合したものである. その機能は, 接着シグナルの伝達と特定の膜環境を保持するフェンス的役割と考えられる.

基底側領域の機能は不明であるが, この部位にHCO_3^-/Cl^- exchangerが存在し, 持続的な酸の分泌を可能にするのであろう. また, エンドサイトーシスされるトランスフェリンレセプターの解析より, 基底側領域の一部の膜は, 波状縁領域に使われる可能性も指摘されている[20,21]. このように骨吸収を行っている破骨細胞は高度に極性化した細胞である. 破骨細胞の骨吸収機能は, それぞれの膜領域が独自の機能を果たすことで維持されるのであろう.

おわりに

以上, 足早にではあったが, 破骨細胞の分化から骨吸収まで順を追って見てきた. この数年の間に, 破骨細胞分化と機能発現の調節機構の解明は, 大きく進歩した. しかし, ①RANKL, OPG, M-CSFの局在のみが, 破骨細胞の形成する部位を決定するのか, ②破骨細胞の分化を決定する細胞内シグナルは何か, ③破骨細胞はなぜ多核化するのか, また関与する分子は何か, ④破骨細胞は骨をどのように認識して骨面に波状縁を形成するのか, ⑤骨吸収に関与する酸や酵素は, どのように波状縁に運ばれるか, ⑥破骨細胞はどのようなシグナルを受けて骨吸収を停止するのか, ⑦破骨細胞のアポトーシスはどのように調節されているのかなど, 多くの問題が未解決である. 今後の研究により, これらの疑問が解明され, 骨粗鬆症をはじめとする骨代謝疾患の治療に貢献すると確信する.

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Comparison of the activities of multinucleated bone-resorbing giant cells derived from CD14-positive cells in the synovial fluids of rheumatoid arthritis and osteoarthritis patients

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Objective. **To investigate the morphology and function of multinucleated bone-resorbing giant cells derived from CD14-positive cells in the synovial fluids (SF) of patients with rheumatoid arthritis (RA) or osteoarthritis (OA).**

Methods. **CD14-positive cells were obtained by magnetic-activated cell sorting of primary cultures of mononuclear cells from the SF. Multinucleated bone-resorbing giant cells were induced from the CD14-positive cells in the presence or absence of cytokines. We examined various characteristics, including osteoclast markers, fusion index and bone-resorption activities of the multinucleated giant cells.**

Results. **Multinucleated giant cells were induced from the CD14-positive cells in the SF of the RA and OA patients by the addition of interleukin (IL)-3, IL-5 and IL-7, or granulocyte–macrophage colony-stimulating factor (GM-CSF), respectively. These multinucleated giant cells were positive for tartrate-resistant acid phosphatase (TRAP), carbonic anhydrase II, actin, vitronectin receptor and the calcitonin receptor. However, the average values for the number of nuclei, fusion index and bone-resorption functions of the SF cells from the RA patients were significantly higher than those derived from the OA patients.**

Conclusion. **These results suggest that the induction and activities of multinucleated bone-resorbing giant cells may play a pivotal role in bone destruction, and that these processes may be enhanced significantly in RA patients.**

Key words: Rheumatoid arthritis, Synovial fluid, CD14-positive cell, Osteoclast.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by invasive synovial hyperplasia, which leads to progressive destruction of the joint. Although the precise mechanism of joint destruction has not yet been elucidated, osteoclasts appear to play a pivotal role in the joint destruction seen in RA patients. Osteoclasts are multinucleated bone-resorbing cells that are derived from CD34-positive haematopoietic stem cells [1–3]. Osteoclasts in the RA joint actively resorb bone at the site at which the proliferating synovial membrane invades the adjacent bone [4]. The osteoclast progenitors are members of the monocyte/macrophage lineage [1], and they differentiate into the mononuclear precursors of osteoclasts (preosteoclasts) [1, 5, 6]. The mononuclear preosteoclasts express tartrate-resistant acid phosphatase (TRAP), which is not produced by peripheral blood monocytes [7]. Mature osteoclasts are generated by the fusion of these mononuclear preosteoclasts, while they are in close contact with stromal cells in the bone marrow [1, 8–10]. Rheumatoid synovial fibroblasts participate in bone destruction by inducing osteoclastogenesis [11–13]. Bone-resorptive cytokines, such as tumour necrosis factor-α (TNFα), interleukin 1 (IL-1), IL-6 and soluble IL-6 receptor (sIL-6R) in the synovial fluid or in the serum are reportedly involved in the immune responses and activation of inflammation seen in RA patients [14–20]. High levels of IL-6 and sIL-6R, IL-17 and fibroblast growth factor (FGF)-2 in the synovial fluids of patients with RA appear to enhance osteoclastogenesis and promote joint destruction [21–23].

Nurse cells were first described in 1980 [24, 25] and are believed to play an important role in the differentiation, maturation and apoptosis of murine thymocytes [26–28]. Thymocytes initially adhere to thymic nurse cells and then crawl underneath them in a process that is referred to as pseudoemperipolesis. We reported previously on the presence of nurse-like cells in the synovial tissues and bone marrow of patients with RA, and suggested an important role for these cells in the pathogenesis of RA [29–31].

Recently, we reported that multinucleated bone-resorbing osteoclast-like cells were generated from peripheral monocytes that differentiated into TRAP-positive mononuclear cells when induced by RA nurse-like cells (RA-NLCs) [32]. In addition, certain cytokines in the synovial fluids (SF) of RA patients are responsible for osteoclast-like cell formation. We detected TRAP-positive mononuclear cells, which differentiated into multinucleated bone-resorbing giant cells, in the SF of patients with RA [32]. Although the presence of the bone-resorbing cells in RA joints is well known, the characteristics and functions of multinucleated bone-resorbing giant cells remain unknown. In the present study, we evaluated differences between RA and OA patients in the

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morphology and function of multinucleated bone-resorbing giant cells, which were derived from CD14-positive monocyte-like cells in their SF.

Patients and methods

Patients

Seven patients with RA (seven women) and five patients with OA (two men and three women) participated in this study. All of the patients were treated at Osaka University Hospital or affiliated facilities. The average ages of the RA and OA patients were 52.8 ± 6.1 and 66.0 ± 6.0 yr, respectively. The diagnosis of RA was based on the 1987 revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) [33], and that of OA was based on clinical and radiological features.

Cell numbers and surface antigen analysis

Synovial fluid was obtained from the knee joints of RA and OA patients by aspiration with an 18-gauge needle under aseptic conditions. Full informed consent was obtained from the patients for sample aspiration and all of the subsequent procedures. The joint-infiltrating cells in the SF of the RA and OA patients were collected by centrifugation at 1900 *g*. The cells were counted using a haemocytometer, whereby dead cells that were stained with trypan blue were excluded.

The surface markers of the cells in the SF samples were examined by staining with monoclonal antibodies (mAbs). In this study, we used fluorescein isothiocyanate (FITC)-conjugated anti-human mAbs that were specific for CD4, CD8, CD15 or CD19 (all from Becton Dickinson, Franklin Lakes, NJ), and phycoerythrin (PE)-conjugated anti-human mAbs that were specific for CD14, CD16 or HLA-DR (all from Becton Dickinson). The mononuclear cells (100 000) were incubated at 4°C for 30 min with 1 mg/ml of the FITC- or PE-conjugated mAbs. After washing twice with phosphate-buffered saline (PBS), the cells were analysed by flow cytometry using FACScan (Becton Dickinson), and the individual cell surface antigens were quantified. Dead cells were eliminated by propidium iodide staining, and excluded from the analysis by setting the scatter gates. The data were analysed using the CellQuest software (Becton Dickinson).

Mononuclear cell culture from the synovial fluid

The joint-infiltrating cells in the SF samples from RA and OA patients were collected as described previously [32]. Briefly, the cells were collected by centrifugation at 1900 *g*, and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) that was supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco BRL) and 100 U/ml of penicillin-streptomycin (Gibco BRL) (Maintenance Medium; MM), and seeded into 6-well flat-bottomed culture plates (Becton Dickinson, Mountain View, CA). The cells were maintained at 37°C in humidified air that contained 7% CO_2, and half of the medium was changed weekly. After 3 to 5 weeks of culture, most of the lymphocytes and granulocytes had disappeared, and the monocyte-like cells that floated on the fibroblast-like cells, which adhered to the bottom of the culture plate, predominated. The non-adherent cells were harvested and the CD14-positive monocyte-like cells were purified from these non-adherent cells using the magnetic-activated cell sorter (MACS; Miltenyi Biotec GmbH, Germany) and magnetic beads that were conjugated with the anti-CD14 antibody, according to the manufacturer's instruction.

Formation of multinucleated bone-resorbing giant cells from CD14-positive monocyte-like cells

A total of 50 000 CD14-positive monocyte-like cells were cultured in MM for 96 h at 37°C and 7% CO_2 in 4-well chamber slides (Lab-Tek Chamber Slide System; Nalge Nunc International, IL), in the presence or absence of the following reagents: recombinant human (rh) interleukin (IL)-3, IL-5 and IL-7; granulocyte–macrophage colony-stimulating factor (GM-CSF); a combination of macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear-factor-κB ligand (RANKL); or in the presence of 10% conditioned medium (CM). The optimal concentration of each cytokine was determined in preliminary experiments.

The frequency of multinucleated giant cell formation was calculated as the fusion index, which has been described previously [34]. Briefly, a minimum of 1000 nuclei within TRAP-positive multinucleated giant cells (>4 nuclei/cell) were counted. The fusion indices of the cells were calculated according to the following formula:

Fusion index (%) = [total number of nuclei within the multinucleated (>4 nuclei/cell) cells/total number of nuclei counted] ×100

Cytochemical and immunocytochemical staining

At the end of culture period, the cells were stained with May-Grunwald–Giemsa and for the tartrate-resistant acid phosphatase (TRAP). TRAP staining was performed with a staining kit (Sigma Chemical Co., St. Louis, MO) in accordance with the manufacturer's instruction. May-Grunwald–Giemsa staining involved a 5-min incubation with a 1:1 dilution of May-Grunwald solution (Merck, Darmstadt, Germany), followed by a 10-min incubation with 1:20 dilution of Giemsa solution (Merck).

The multinucleated giant bone-resorbing cells, which differentiated from CD14-positive monocyte-like cells, were fixed with cold acetone, and stained immunocytochemically with rabbit polyclonal antibodies that were specific for actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), carbonic anhydrase II (Rockland, Gilbertsville, PA) or the vitronectin receptor (Chemicon International Inc., Temecula, CA), or with a goat polyclonal antibody that was specific for the calcitonin receptor (Santa Cruz Biotechnology).

Cytokines and reagents

rhIL-3, IL-5, GM-CSF and M-CSF were purchased from R&D Systems (Minneapolis, MN). rhIL-7 was obtained from Genzyme Corporation (Cambridge, MA), and the receptor activator of nuclear-factor-κB ligand (RANKL) was purchased from Peprotech (London, UK). CM were prepared as reported previously [35]. Briefly, a mixture of peripheral blood mononuclear cells (PBMC) from 10 healthy donors was stimulated with phytohaemagglutinin (PHA; Sigma) at 37°C for 72 h. The culture supernatant fluids were collected, filtered and used as conditioned media.

Bone resorption assays

To determine the resorption activities of the TRAP-positive giant cells, 70 000 CD14-positive monocyte-like cells were cultured on dentin slices that were placed in 4-well chamber slides in medium with different cytokines or CM for 14 days. The cells on the dentin slices were removed by brushing in distilled water, cleaned by ultrasonication to remove adherent cells, and stained with haematoxylin (Sigma). The resorption pits were counted under a microscope. As an alternative method for analysing bone resorption, the CD14-positive monocyte-like cells were cultured on

calcium phosphate-coated discs (Osteologic; Millenium Biologix Inc., Ontario, Canada) using the culture conditions described above. After 14 days of incubation, the discs were washed in 6% NaClO and 5.2% NaCl to remove the cells, dried, and examined by phase-contrast microscopy. The resorbed area on each disc was measured using the MacSCOPE image analyser (Mitani Corp., Fukui, Japan).

Statistical analysis

The values are presented as the means ± standard deviation (S.D.). Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test. *P* values of >0.05 were considered to be statistically significant.

Results

Numbers and cell surface markers of joint-infiltrating cells in the RA and OA patients

In order to evaluate the absolute number of joint-infiltrating cells in the SF, the cells were collected and counted. The average number of cells in the SF of the RA patients was $11 \pm 7.8 \times 10^5$/ml, and that of the OA patients was $7.3 \pm 3.9 \times 10^4$/ml. There was a significant increase in the average number of cells in the SF of RA patients, as compared with the SF of OA patients ($P < 0.01$). FACScan analysis was performed to examine the cell surface phenotype of the joint-infiltrating cells. The cells in the RA and OA SF samples were positive for HLA-DR, and weakly positive for CD4, CD8 and CD16. The percentage of CD4-positive cells in the RA-SF was significantly higher than in the OA-SF (RA-SF, 39.2 ± 8.6% *vs* OA-SF, 18.1 ± 9.3%; $P < 0.05$). The only significant difference between the RA and OA patients was in the levels of CD4-positive cells in their SF.

Differentiation of cultured CD14-positive monocyte-like cells into multinucleated bone-resorbing giant cells

The non-adherent cells were harvested after 4 weeks of primary culture, and the CD14-positive cells were collected using MACS with magnetic beads that were conjugated to the anti-CD14 antibody. The purity of the CD14-positive cells was >98%, as assessed by FACS analysis (data not shown). The CD14-positive monocyte-like cells from the RA-SF and OA-SF samples were cultured with IL-3 in the absence of fibroblast-like cells. These cells differentiated into multinucleated bone-resorbing giant cells (Fig. 1A and C). However, the numbers of cell nuclei differed among the two groups of patients. We counted the nuclei in all of the multinucleated cells that had five nuclei or more. The average number of nuclei was significantly higher in the RA group (RA, 24.66 ± 6.06 nuclei *vs* OA, 11.2 ± 2.61 nuclei; $P < 0.05$) (Fig. 2). These multinucleated cells were positive for TRAP (Fig. 1B and 1D), and resorption pits were observed on dentin slices (Fig. 3A and B) and on Osteologic discs (Fig. 4A and B). The multinucleated cells were positive for carbonic anhydrase II, actin, vitronectin receptor and calcitonin receptor (Fig. 5). The average positive percentage of calcitonin receptor in TRAP-positive multinucleated cells was 90.69 ± 7.2%, and TRAP-positive mononuclear cells were calcitonin receptor negative. IL-5, IL-7, GM-CSF and a combination of RANKL and M-CSF also induced the differentiation of CD14-positive monocyte-like cells into multinucleated cells. These cells were also positive for TRAP, and had the same functions and characteristics (data not shown). In the presence of each of the cytokines, the fusion index of the multinucleated cells, which were derived from the CD14-positive monocyte-like cells from the RA-SF, was significantly higher than that of the OA-SF ($P < 0.05$). Interestingly, the CD14-positive cells that were cultured with a mixture of RANKL and M-CSF exhibited lower fusion indices than cells that were treated with IL-3 (Fig. 6). These results were confirmed in three separate experiments, using various concentrations of the cytokines. The

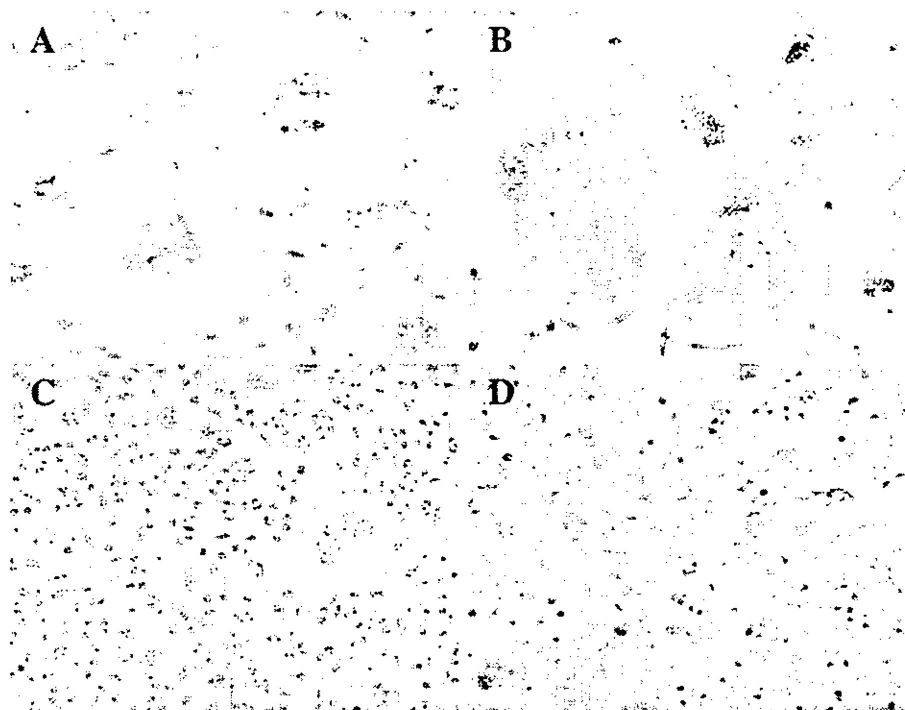

FIG. 1. Morphological examination of multinucleated giant cells that were derived from CD14-positive cells from the RA-SF or OA-SF. The CD14-positive monocyte-like cells were cultured with IL-3 (1 ng/ml). (A, C) May-Grunwald–Giemsa staining and (B, D) TRAP staining of multinucleated giant cells. Multinucleated giant cells from the RA-SF were bigger and had more nuclei compared with those from the OA-SF. Original magnification ×100.

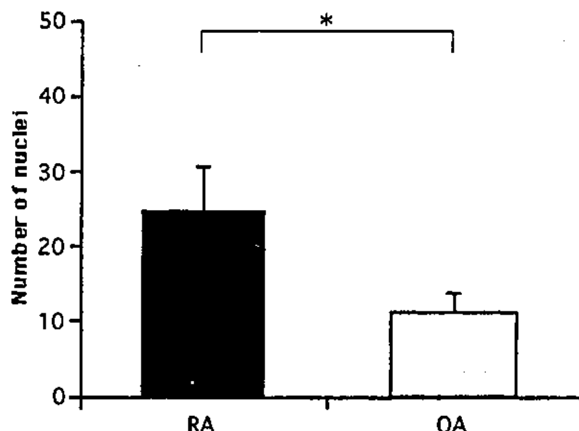

FIG. 2. The number of the multinucleated giant cells was counted. The nuclei in all of the multinucleated cells with five nuclei or more were counted. The average number of nuclei was significantly higher in the RA group. $*P < 0.05$ *vs* OA.

multinucleated cells were induced by those cytokines in a dose-dependent manner and then the effect became fixed both in RA and OA.

Number of resorption pits on the dentin slices, and the percentage resorption on Osteologic discs

In order to determine the ability of multinucleated cells to absorb bone, the CD14-positive monocyte-like cells were cultured in medium with IL-3 for 14 days on either dentin slices or Osteologic discs. After the incubation period, the multinucleated giant cells from CD14-positive monocyte-like cells in the IL-3-stimulated RA-SF and OA-SF samples formed resorption pits on the dentin slices. The number of resorption pits formed by the RA-SF was significantly higher (143.0 ± 19.52; $P < 0.05$) than that formed by the OA-SF (9.0 ± 2.0) (Fig. 7). Since all of the cultures formed resorption pits on the Osteologic discs, the percentage resorption was calculated as the area of resorption relative to the total surface area of the disc. A marked increase in lacunar resorption was noted for the RA-SF cultures, in which the extent of resorption was $46.04 \pm 6.39\%$, as compared with that of the OA-SF cultures ($12.38 \pm 2.18\%$; $P < 0.05$) (Fig. 8). When the CD14-positive cells were purified from RA-SF and OA-SF which does not pre-culture for 4 weeks, they did not form resorption pits on either dentin slices or Osteologic discs (data not shown).

Discussion

In this study, we demonstrated that multinucleated bone-resorbing giant cells were induced from CD14-positive monocyte-like cells in both RA-SF and OA-SF. However, the fusion indices and functional parameters of multinucleated cells that were derived from CD14-positive monocyte-like cells were increased in the RA-SF. While the percentages of monocyte/macrophage cells were similar, the absolute numbers of those cells were significantly higher in RA-SF than in OA-SF. These results suggest that the RA-SF contains many more cells with the ability to differentiate into TRAP-positive preosteoclasts than are found in the OA-SF. The resorption pits on either dentin slices or Osteologic discs of multinucleated cells that were derived from CD14-positive

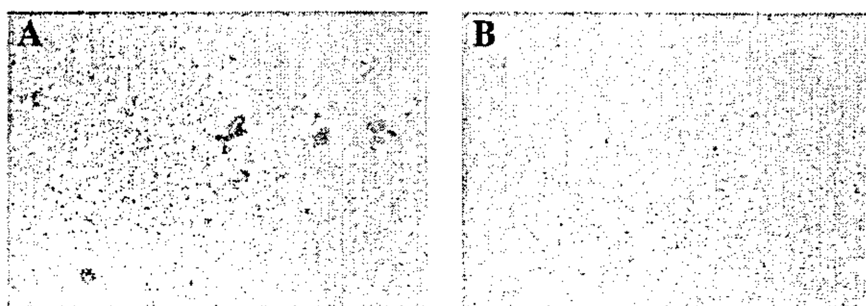

FIG. 3. Examination of resorption pits formed by multinucleated bone-resorbing giant cells on dentin slices. The CD14-positive monocyte-like cells were cultured with IL-3 (1 ng/ml) for 14 days on dentin slices. (A) Resorption pits were observed on dentin slices by bone-resorbing giant cells which were derived from CD14-positive cells from the RA-SF, but (B) not observed from the OA-SF. Original magnification ×200.

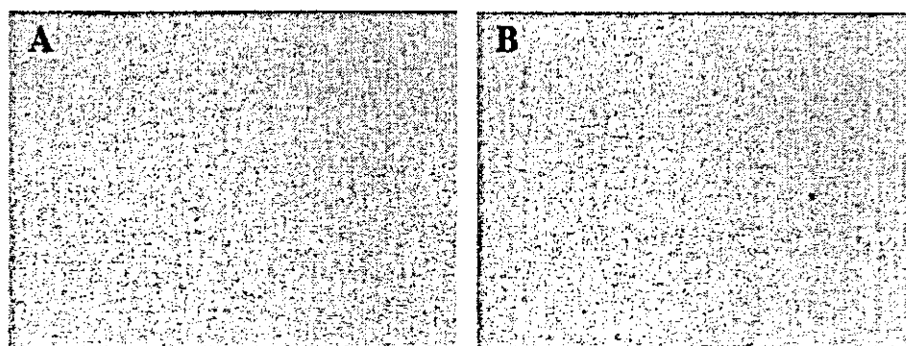

FIG. 4. Examination of resorption area formed by multinucleated bone-resorbing giant cells on Osteologic discs by using phase-contrast microscopy. The CD14-positive monocyte-like cells were cultured with IL-3 (1 ng/ml) for 14 days on Osteologic discs. (A) A wide-ranging area was formed by bone-resorbing giant cells which were derived from CD14-positive cells from the RA-SF, but (B) not formed in OA-SF. Original magnification ×100.

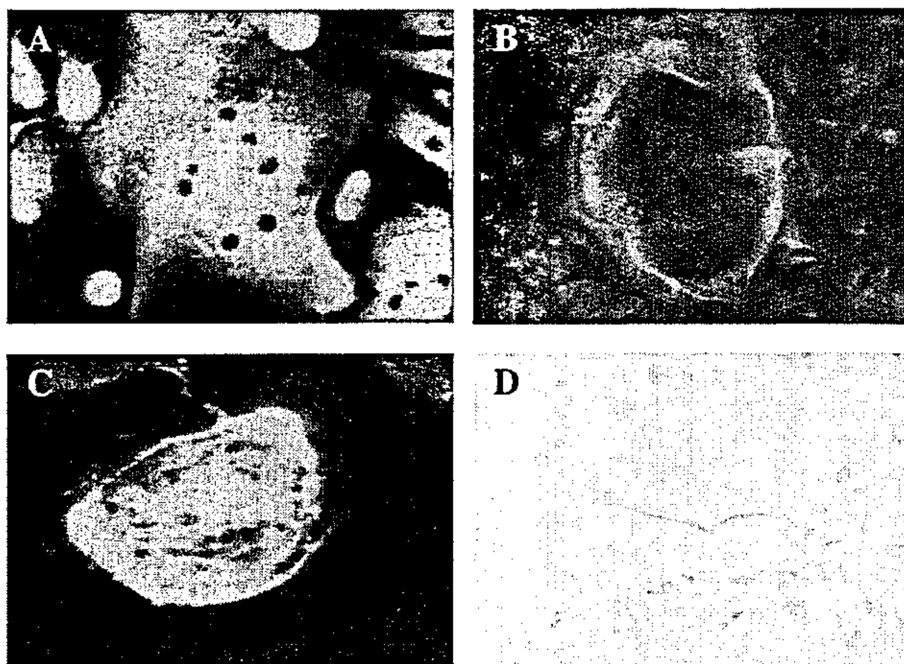

FIG. 5. Immunocytochemical examination of multinucleated giant cells which were derived from CD14-positive cells from the RA-SF. The CD14-positive monocyte-like cells were cultured with IL-3 (1 ng/ml). Multinucleated giant cells were positive for (A) carbonic anhydrase II, (B) actin, (C) vitronectin receptor, (D) calcitonin receptor. Original magnification ×200.

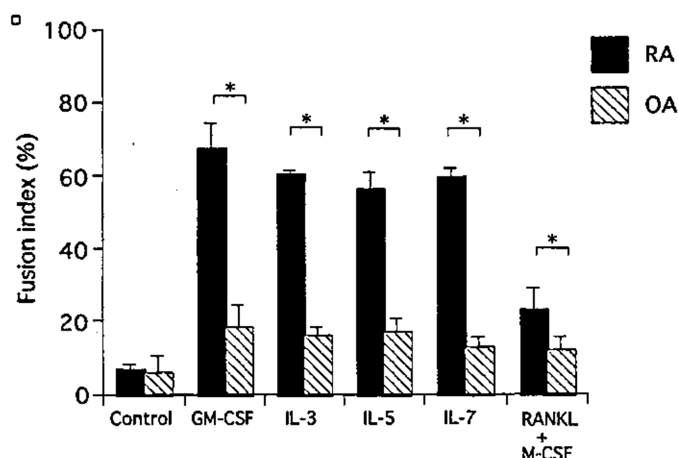

FIG. 6. IL-3, IL-5, IL-7, GM-CSF and a combination of RANKL and M-CSF induced the differentiation of CD14-positive monocyte-like cells into multinucleated cells. $*P<0.05$ *vs* OA.

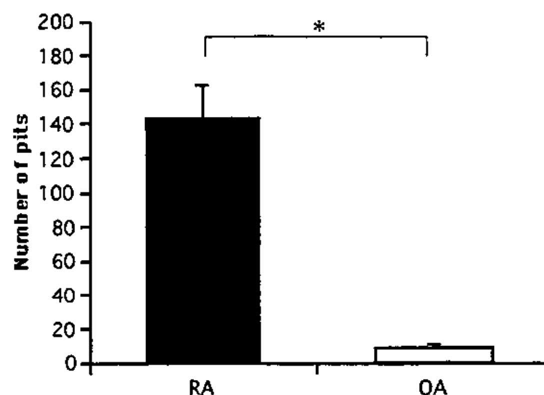

FIG. 7. Comparison of capability of resorption pit formation on dentin slices between bone-resorbing giant cells which were derived from CD14-positive cells from the RA-SF and OA-SF. The number of resorption pits was counted under microscopic examination. The number of resorption pits formed by the RA-SF was significantly higher than that formed by the OA-SF. $*P<0.05$ *vs* OA.

monocyte-like cells were increased in the RA-SF. In these functional parameters, high bone-resorbing activity of multinucleated cells in the RA-SF may be related to the survival rate or the number of multinucleated cells.

We reported previously that CD14-positive monocyte-like cells could be induced and maintained in the presence of nurse-like cells (RA-NLC), which were isolated from RA synovial tissue and bone marrow [32]. These cells expressed TRAP activity, and differentiated into multinucleated bone-resorbing giant cells when stimulated with IL-3, IL-5, IL-7 or GM-CSF in the absence of accessory cells. The RA-NLCs were shown to play an important role in the differentiation and maturation of lymphocytes through pseudoemperipolesis in RA joints [29–31]. In the primary cultures of SF mononuclear cells, the relatively large, round-shaped, non-adherent cells and adherent cells predominated after 3 weeks, and some of these adherent cells had nursing activities (H. Takano, personal communication), which suggests that adherent cells with nursing activities may play a role in the osteoclastogenesis observed in this study.

The multinucleated bone-resorbing cells shown in this study differentiated in the presence of IL-3, IL-5, IL-7 and GM-CSF. These cytokines and growth factors are known to promote the proliferation and differentiation of primitive haematopoietic cells. IL-3, IL-5 and GM-CSF are produced by activated T cells, and their receptors contain a common beta subunit [36]. In the present study, we found that CD4-positivity was more prevalent in the RA-SF than in the OA-SF, which suggests that T cells accumulate and produce IL-3, IL-5 and GM-CSF in the RA synovial fluid. GM-CSF is often detected in the joints of RA patients [37, 38], and synovial RA-NLCs produce GM-CSF *in vitro* [29, 30]. Matayoshi *et al.* [3] reported that both IL-3 and GM-CSF induced the differentiation of haematopoietic precursor cells into osteoclasts in the absence of stromal cells [3].

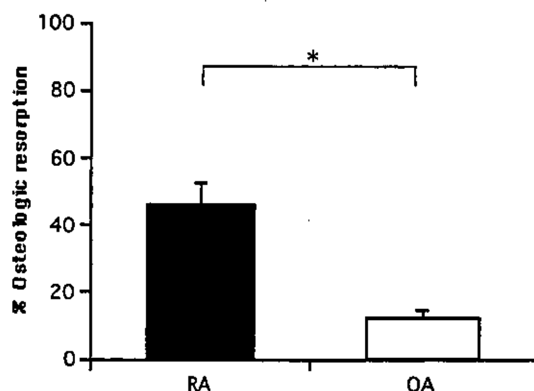

FIG. 8. Comparison of capability of resorbing area on Osteologic discs between bone-resorbing giant cells which were derived from CD14-positive cells from the RA-SF and OA-SF. The resorbed area on each disc was measured using the MacSCOPE image analyser. A marked increase in lacunar resorption was noted for the RA-SF cultures as compared with that of the OA-SF cultures. $*P < 0.05$ *vs* OA.

RANKL has been reported as a potent inducer of osteoclast development from monocytes, and is a key molecule in osteoclastogenesis [39, 40]. However, it was very interesting to note that a mixture of RANKL and M-CSF exhibited weaker induction of multinucleated cells than IL-3, IL-5, IL-7 and GM-CSF in our experiments. This finding suggests that stimulation of IL-3, IL-5, IL-7 and GM-CSF at the step of fusion of the pre-cultured CD14-positive monocyte-like cells is more dominant than RANKL. However, it is likely that RANKL is participating in differentiation of CD14-positive monocyte-like cells into preosteoclasts during the co-culture with fibroblast-like cells.

In this study, the fusion indices and functional parameters of the multinucleated cells, which were derived from CD14-positive monocyte-like cells, were much higher in the RA-SF than in the OA-SF. This result concurs with the conclusions of previous reports, in which histochemical studies indicated that TRAP-positive multinucleated cells were more numerous in the RA synovium than in the OA synovium [21, 41, 42]. In this study, similar numbers of CD14-positive cells were cultured from the SF of RA and OA patients, but there were significant differences between the RA-SF and OA-SF in terms of fusion indices and bone resorption activities. Our results suggest that the functions of CD14-positive cells may be enhanced in RA due to the enhanced ability of RA-SF stromal cells with nursing activity to support the differentiation of monocyte-like cells into TRAP-positive preosteoclasts. A detailed investigation of the differences in CD14-positive cell populations between RA and OA patients is underway.

In conclusion, CD14-positive cells and activated T cells in the RA-SF may play important roles in RA pathogenesis, which is characterized by progressive bone destruction and the enhanced function of haematopoietic cells, such as preosteoclast-like cells. These discoveries may provide a tool for understanding the mechanisms of bone destruction in RA, and for the development of effective treatments for joint destruction in RA patients.

The authors have declared no conflicts of interest.

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Evidence for existence of oligoclonal tumor-infiltrating lymphocytes and predominant production of T helper 1/T cytotoxic 1 type cytokines in gastric and colorectal tumors

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Abstract. Tumor-infiltrating lymphocytes (TIL) play a central role in cellular immunity against tumor. We have revealed the characteristics of TILs in terms of T-cell receptor (TCR) repertoire, T-cell clonality, and cytokine production. TCR repertoire analyses and CDR3 size spectratyping were performed using peripheral blood mononuclear cells (PBMCs) and tissue specimens of gastric or colorectal cancers surgically resected from 11 patients. The cytokine expression was measured by real-time quantitative polymerase chain reaction. TCR repertoires were similar among multiple tissue specimens from different sites of the same tumor. Similar peak patterns of CDR3 size spectratyping were observed among these tumor specimens, but not in normal tissues or PBMCs. In addition, identical peaks were detected in multiple specimens of the same tumor. The ratio of the levels of IFN-γ to that of IL-4 is significantly higher for tumor lesions compared with PBMCs. These results suggested that a limited number of TILs locally expand in response to tumor antigens exiting within gastric or colorectal cancers and local predominant production of the T helper 1/T cytotoxic 1 type cytokine may affect the anti-tumor immune response of TILs.

Introduction

The host immune system recognizes tumor cells and tries to reject tumors such as melanoma. In the anti-tumor response, tumor-infiltrating T lymphocytes (TIL) are known to play a significant role (1). TILs are enriched with T-cells, which recognize antigens expressed on the surface of autologous tumor cells (2). Differential antigens such as Melan-A/MART-1 (3-5), tumor-associated antigens (6) or mutated self-antigens (7) have been defined as tumor-specific antigens. These antigens or antigen peptides specific for tumors have been developed for cancer vaccines, which can induce anti-tumor-specific T-cells. Many approaches to therapeutic cancer vaccine development have been under clinical trial.

In many human cancers other than melanoma, the anti-tumor response mediated by T-cells occurs in limited local sites around the tumor, although the immune response in no way controls tumor growth. Gaudin *et al* has already described local expansion of T-cell clones in renal cell carcinoma (8). However, little is known about whether TILs respond to tumor antigens in patients with gastric or colorectal cancer and what the functional characteristics of the TILs are. Proliferation of T-cells specific for tumor antigens can skew TCR repertoires in local sites around the tumor. Determination of the usage of TCR repertoires at local sites allows us to demonstrate that the existence of TILs that can exert an anti-tumor immune response and enable us to clarify the characteristics of TILs. T-cells recognize a peptide antigen present on the surface of a major histocompatibility antigen (MHC) molecule (9). Therefore, TILs that recognize a common antigen are thought to express the same or a similar T-cell receptor. Analysis of the specific T-cell receptor (TCR) of TIL can provide information on the nature of the antigen(s) recognized by TIL. If a common tumor antigen can be defined, it should enable us to effectively induce an anti-tumor immune response for therapy.

Cytotoxic T-cells play an important role in cellular immunity against tumors. It has been reported that CD8 positive anti-tumor T-cells infiltrate local lesions of human colorectal tumor tissue (10) and renal cell carcinoma (11). Furthermore, cytokines are key molecules that modulate the

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function of T-cells. T helper type 1 cells (Th1), Th2, T cytotoxic 1 cells (Tc1), and Tc2 play immunoregulatory roles (12,13). Alterations of T cytotoxic type 1 (Tc1)/T cytotoxic type 2 (Tc2) subsets may cause host anti-tumor immune responses (14-16). Although cytokine expression patterns in TILs isolated from various tumor cell types have been reported, the changes of cytokine production in TILs are still somewhat controversial.

To elucidate the characteristics of T-cells infiltrating tumor tissues, we analyzed TCR variable region repertoires within tissue specimens from surgical lesions of patients with gastric or colorectal cancer. In addition, we analyzed T-cell clonalities by CDR3 size spectratyping with these tissues. We found similar TCR repertoires among multiple specimens from different sites of the same tumor, suggesting that the tumor-specific T-cells recognize common antigens in tumor lesions. Furthermore, we examined the expression levels of several cytokines such as IFN-γ and IL-4 by real-time quantitative polymerase chain reaction. The elevated levels of IFN-γ/IL-4 were observed in tumor lesions compared to PBMCs. The results suggest that polarization of TIL toward Th1/Tc1 subsets has an effect on the anti-tumor immune response.

Table I. Patient characteristics.

Patient	Age	Sex	Diagnostic	Stage	Recurrent
1	64	M	Sigmoid colon	2	-
2	73	M	Gastric	1b	-
3	53	M	Sigmoid colon	4	-
4	68	M	Gastric	3a	+
5	65	M	Gastric		+
6	68	M	Transversum Colon	3a	-
7	73	M	Rectal	2	-
8	33	M	Rectal	3b	-
9	74	M	Rectal	2	-
10	70	F	Rectal	2	+
11	50	M	Rectal	3a	-

Patients and methods

Patients and samples. Tissue specimens and peripheral blood mononuclear cells (PBMCs) were collected from 11 patients who underwent surgical operations at Tohoku University Hospital (Table I). The tissues were freshly collected from tumor specimens immediately after the surgical operations. Fresh specimens were collected from one or more different sites of tumor resected from the same patient. Each of the specimens was obtained from a location distant from the others by punch biopsy. The tissues were immediately frozen in liquid nitrogen for RNA extraction. Tissue samples in which lymphocyte infiltration was verified by histological testing were used for analyses. Normal tissue samples around tumor, in which few lymphocyte invasions were found, were used as a control. PBMCs were collected at the time of the operations. All samples used in the present study were collected after informed consent had been obtained.

TCR repertoire analysis. The methods for isolation of RNA from PBMCs and adaptor ligation-mediated polymerase chain reaction (PCR) were previously reported (17,18). Freshly isolated RNA was converted to double-stranded cDNA using Superscript cDNA synthesis kits (Invitrogen, CA) according to the manufacturer's instructions, except that a specific primer (BSL-18) was used. The P10EA/P20EA adaptors were ligated to the 5'-end of cDNA and this adaptor-ligated cDNA was cut with *NotI*. PCR was performed with T-cell receptor α-chain constant region (TCRAC)-specific or T-cell receptor β-chain constant region (TCRBC)-specific primers (CA1 or CB1) and P20EA. The second PCR was performed with CA2 or CB2 and P20EA. The third PCR was performed using both P20EA and 5'-biotinylated CA4 or CB4 primer for biotinylation of PCR products. TCRAV and TCRBV repertoires were analyzed by microplate hybridization assay (MHA) (17,18). In short, 10 pmol of amino-modified oligonucleotides specific for TCRAV and TCRBV segments were immobilized onto carboxylate-modified 96-well microplates (C type, Sumitomo Bakelite, Tokyo, Japan) with water-soluble carbodiimide. Prehybridization and hybridization were performed in GMC buffer (0.5 M Na_2HPO_4, pH 7.0, 1 mM EDTA, 7% SDS and 1% BSA) at 47°C. Sixty microliters of denatured 5'-biotinylated PCR products mixed with an equivalent volume of 0.4 N NaOH/10 mM EDTA was added to 6 ml of GMC buffer. Hybridization solution, 100 μl, was used in each well of the microtiter plate containing immobilized oligonucleotide probes specific for the V segment. After hybridization, the wells were washed 6 times with washing buffer (2X SSC, 0.1% SDS) at room temperature and then with VA (0.4X SSC, 0.1% SDS) or VB (0.6X SSC, 0.1% SDS) stringency washing buffer for 10 min at 37°C. The blocking of non-specific binding was done with 200 μl of TB-TBS buffer [10 mM Tris-HCl, 0.5 M NaCl, pH 7.4, 0.5% Tween 20 and 0.5% blocking reagent (Roche Diagnostics, Germany)]. Next, 100 μl of a 1:1000-diluted, alkaline phosphatase-conjugated streptavidin (Invitrogen) in TB-TBS was added, and the sample was incubated at 37°C for 30 min. The plates were washed 6 times in T-TBS (10 mM Tris-HCl, 0.5 M NaCl, pH 7.4, 0.5% Tween 20). For color development, 100 μl of substrate solution (4 mg/ml p-nitro-phenylphosphate, Sigma, in 20% diethanolamine, pH 9.8) was added, and then absorbance was determined at 405 nm.

T-cell clonality analysis with CDR3 size spectratyping. PCR for CDR3 size spectratyping was performed for 30 cycles in a 20-μl volume under the same conditions as described above. PCR was performed with 1 μl of the second PCR product, 0.1 μM of 5'-Cy5 CA2/CB2, and 0.1 μM primer specific for each variable segment. The oligonucleotide probes for hybridization were used as primers specific for each variable segment. Five microliters of 1:20 or 1:50 diluted PCR product in dye solution (95% formamide, 10 mM EDTA and 0.1% blue dextrane) was analyzed in 6% denatured acrylamide gel with an ALFred sequence analyzer (Pharmacia Biotech, Uppsala, Sweden). The data obtained were transferred to Fragment Manager Software (Pharmacia Biotech). As a

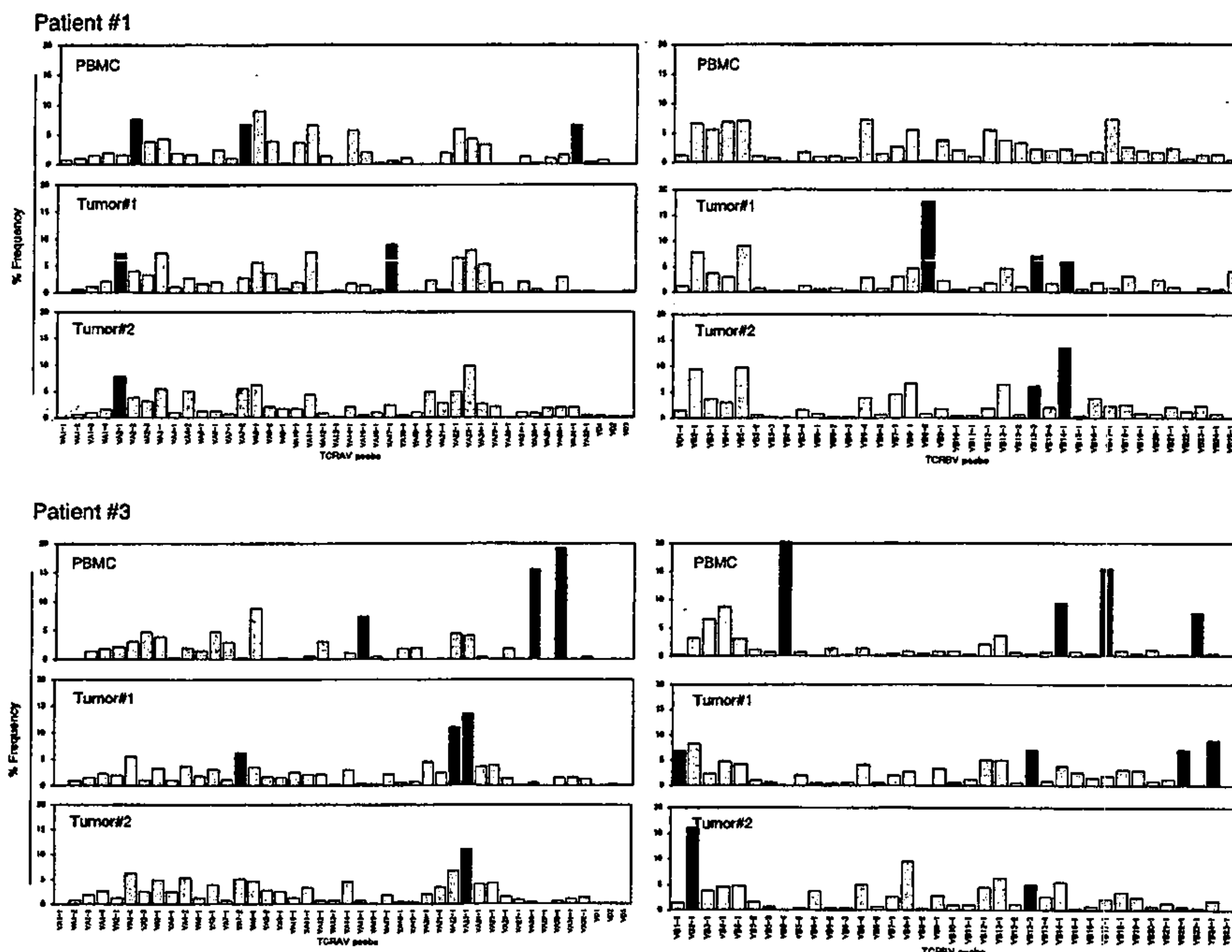

Figure 1. Representative results of T-cell receptor (TCR) variable region α-chain (TCRAV) and TCR β-chain (TCRBV) repertoires in patients with gastric or colorectal cancer. TCRAV (left) and TCRBV (right) repertoires were analyzed with a peripheral blood mononuclear lymphocyte (PBMC) and two samples from different sites of the same tumor. Percent frequencies of expression levels in each segment are shown. The segments, in which a high level of expression above the control levels was found, are shown by closed bars.

control, PBMCs from 10 healthy donors were also analyzed for the peak patterns, revealing multiple peaks as Gaussian patterns with 3-nucleotide intervals in each V segment.

Quantification of cytokine mRNA. The expression levels of mRNA of interleukin-2 (IL-2), interferon-γ (IFN-γ), interleukin-4 (IL-4), interleukin-5 (IL-5), and tumor necrosis factor-α (TNF-α) were examined for PBMCs, normal tissues and tumor lesions of patients with real-time quantitative polymerase chain reaction (PCR) using GeneAmp 5700 Sequence Detector (Applied Biosystems, CA). Freshly isolated RNA was converted to cDNA using Superscript™ reverse transcriptase II (Invitrogen, CA). Next, one-tenth of the cDNA was amplified with qPCR Mastermix for Sybr™ Green I (Eurogentec, Belgium) according to the manufacturer's instructions. Primer pairs specific for each cytokine gene were used (19). The C_T, or threshold cycles, values were obtained with 10-fold dilution of PCR products ranging from 20 to 0.0002 amol/ml within the exponential phase of the PCR. For standard curves, the C_T values were plotted against the known amount of PCR products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used for the internal control. The results were expressed as the ratio of the amount of the respective cytokine-specific cDNA to the amount of GAPDH-specific cDNA.

Determination of nucleotide sequence of CDR3 regions. PCR was performed with a forward primer specific for the variable region (VA7-2 or VB13-2) and a reverse primer specific for the constant region (CA4 or CB4) under conditions described above. Primer used in this study was as follows: VA7-2-: TTCCTTAGTCGGTCTAAAGGG, VB13-2: GAATTTCCT GCTGGGGTTGG, CA4: ATAGGCAGACAGACTTGTCA CTG, CB4: ACACCAGTGTGGCCTTTTGGGTG. After the PCR products were eluted from agarose gel, the PCR products were cloned into pCR2.1-TOPO vector with TOPO TA Cloning kit (Invitrogen, CA). DH5α-T1® competent cells were transformed with the recombinant plasmid DNA. Sequence reaction were performed with BigDye® Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, CA) and analyzed by ABI PRISM 3100 genetic analyzer (Applied Biosystems).