在するとされているので，INH および RFP の 2 剤ならば有効な治療を行えるが単剤治療を行うと容易に耐性菌が増殖することが分かる．

当初感受性であった結核患者が，不適切な治療（単剤治療，薬剤濃度の不足，服薬コンプライアンスの不良など）により耐性菌が誘導されて生じたものを，獲得耐性（既治療耐性，acquired resistance）と呼ぶ．こうして生じた耐性結核患者からの感染対策が適切になされないと，耐性結核菌による感染が生じ，それまで抗結核薬治療を受けたことがないにもかかわらず当初から薬剤耐性を示す結核患者が発生することになる．これを初回耐性（未治療耐性，primary resistance）と呼ぶ．以上から分かるように，獲得耐性率は個々の結核患者に対する治療の適切さを，初回耐性率は結核感染対策の適切さを，それぞれ反映していると考えられる．

我が国および諸外国における多剤耐性結核の現状

世界保健機関（WHO）および国際結核肺疾患予防連合（IUATLD）は，1999 年から 2002 年の間に結核菌の耐性率についてのサーベイランスを全世界で行っており，その結果を 2004 年に発表している[1]．未治療患者における初回多剤耐性率は，カザフスタンとイスラエルが 14.2％ と最も高く，その他旧ソ連のトムスク州，エストニア，リトアニア，ラトビア，中国の遼寧省，河南省などで高かった．また，既治療患者における獲得多剤耐性率は，オマーンが 58.3％，カザフスタンが 56.4％ と最も高かった．豊かな国で治療についてのプログラムが確立している国では耐性率は低く，また貧しい国で薬剤がほとんど使用できない国でも耐性率は低い．中進国で薬剤が広く使用されているが適切な治療プログラムが確立していない国で耐性率が高くなることになる[2]．

我が国での耐性率については，結核療法研究協議会が定期的に全国的なサーベイランスを行っている．1997 年の調査によれば，初回多剤耐性率は 0.8％，獲得多剤耐性率は 19.7％ であった[3]．1992 年の調査と比較するとやや上昇傾向がみられるが，薬剤感受性試験の方法が絶対濃度法から比率法に変更されているため単純な比較はできない．

多剤耐性結核の診断

かつて我が国での結核菌の薬剤感受性試験は絶対濃度法で行われ，各薬剤に複数濃度が設定されており解釈が困難であったが，現在は世界的な標準法に近づけるべく比率法が用いられるようになった．これは，100 倍希釈菌液と 1 万倍希釈菌液を用意し，100 倍希釈菌液を薬剤含有培地に接種して 1 万倍希釈菌液を接種した対照培地と比較し，薬剤含有培地のコロニー数が多ければ耐性菌の割合が 1 % 以上であり耐性であると判定するものであり，絶対濃度法に比して明確に判定できるようになった．さらに，各薬剤の濃度を 1 濃度として解釈に混乱を来さないようにした．ただし，INH のみ試験濃度を 0.2，1.0 (μg / ml) の 2 濃度としているが，耐性の判定には 0.2 μg / ml を用いる．1.0 μg / ml の成績は多剤耐性例で使用可能薬剤に乏しい場合にのみ参考とする．

近年 MGIT 法や最小阻止濃度（MIC）測定によるプロスミック MTB－I 法など，液体培地を用いた薬剤感受性試験が普及しつつあり，これらによれば 1 週間程度で結果を得られるので，米国疾病対策センター（CDC）の「30 日以内に感受性結果を報告すること」との勧告を満たすことができる．さらに最近では，分子生物学的手法により耐性遺伝子の変異を検出する方法が確立しつつある．特に RFP 耐性はほとんどが *rpoB* 遺伝子の変異によるものであるため有用性が高い．培養された結核菌だけではなく，喀痰などの臨床検体から直接 *rpoB* 遺伝子変異を検出する方法も検討されており[4]，いずれは診断・治療開始時に直ちに感受性結果を得ることが可能になるかも知れない．

多剤耐性結核の治療，治療成績

抗結核薬は，その抗菌力と安全性に基づき，First－line drugs と Second－line drugs に分けられている（表 1）が，中でも INH，RFP の 2 剤が抜きん出て重要な薬剤であることは論を待たず，多剤耐性結核の定義が「INH，RFP の 2 剤に耐性である結核」であることにそれが表れている．多剤耐性結核が感受性結核に比べて治療が困難になるのは当然であり，これまでの治療成績の報告をみると，おおむね 50 ～ 85 % 程度の治療成功率と報告されている[5~10]．近年の報告

表1 成人の標準投与量と最大投与量（文献[11]より引用）

薬剤名	略号	標準量 (mg / kg / 日)	最大量 (mg / body / 日)
First－line drugs（a）			
リファンピシン	RFP	10	600
イソニコチン酸ヒドラジド	INH	5	300
ピラジナミド	PZA	25	1,500
First－line drugs（b）			
ストレプトマイシン	SM*	15	750（1,000）
エタンブトール	EB**	15（25）	750（1,000）
Second－line drugs			
カナマイシン	KM*	15	750（1,000）
エチオナミド	TH***	10	600
エンビオマイシン	EVM****	20	1,000
パラアミノサリチル酸塩	PAS	200	12 g / 日
サイクロセリン	CS	10	500
レボフロキサシン	LVFX*****	8	600

*：SM，KM の投与量は毎日投与の場合の投与量である．最初の2ヵ月以内は毎日投与しても可．SM 週2回，KM 週3回の場合は1日最大投与量を 1 g / body とする．

**：EB は最初の2ヵ月間は 25 mg / kg（1,000 mg / 日）を投与してもよい（視力障害に注意）．ただし，3ヵ月目以後も継続投与する場合には 15 mg / kg（750 mg / 日）とする．

***：TH は 200 mg / 日より漸増する．

****：EVM は最初の2ヵ月間は毎日，以後は週2～3回投与する．

*****：LVFX は抗結核薬としては未承認である．RFP または INH が投与不可の場合に限り，感受性であれば感受性のある他の抗結核薬との併用も考慮する（ただし，小児や妊婦は禁忌）．

表は上から下に優先選択すべき薬剤の順に記載されている．なお，SM，KM，EVM の同時併用はできない．抗菌力や交差耐性などから，SM → KM → EVM の順に選択する．

ではやや予後が改善されているが，良好となる因子としては，ニューキノロン剤の使用，外科的手術の併用などが挙げられている．ただ，長期的な再発についての報告は乏しく，また副作用のある薬剤をより多くより長期に使用する必要があることから治療脱落率も高くなる．

多剤耐性結核の治療は，陰圧病室を備え外科的治療も可能な専門施設で行うべきである．重要なのは，これが治癒に導けるラストチャンスであるという認識を持つことである．すなわち，① 使用可能な薬剤をできるだけ多く投与する，② 手術の適応を積極的に考慮する，

③ 軽微な副作用では薬剤を中止しない，④ 優先的に直接服薬確認治療（DOTS）の対象とし服薬が確実に行われるようにする，などである．この段階での不適切な治療のために，患者本人の人生にとりかえしのつかない結果を招く可能性があることを認識しなければならない．

治療にあたっては，日本結核病学会治療委員会が原則を提示している[11]．すなわち，

① 治療当初は投与可能な感受性のある薬剤の中から最低でも3剤（可能なら4～5剤）を菌陰性化後6ヵ月間投与し，その後は長期投与が困難な薬剤を除き，さらに菌陰性化後24ヵ月間治療を継続する．

② 結核菌の薬剤耐性化は遺伝子の点突然変異によるため，菌は薬剤に対し一定の確率で耐性化する．このため，感受性のある一剤のみの変更は容易にその薬剤の耐性獲得に帰結するため禁忌であり，治療薬を変更する場合は一挙に複数の有効薬剤に変更する．

③ 薬剤の選択は別表（表1）の記載順に従って行う．ただし，ストレプトマイシン（SM），カナマイシン（KM），エンビオマイシン（EVM）は同時併用はできない．抗菌力や交差耐性を考慮し，SM→KM→EVMの順に選択する．また，フルオロキノロンも同時併用はできない．抗菌力や副作用などから，レボフロキサシン→シプロフロキサシン→スパルフロキサシンの順に選択する．

④ 外科治療が可能な患者では治療当初から外科療法を積極的に考慮する．なお，外科治療の成功のためにも，幾つかの有効な抗結核薬が不可欠である．

⑤ 多剤耐性のうち，INH 0.2 μg/ml 耐性，1 μg/ml 感受性の場合はINHを投与してもよいが，有効薬剤には数えない．

表1の薬剤だけでは治療困難な場合の選択薬として，WHOの耐性結核ガイドラインではクロファジミン，アモキシシリン/クラブラン酸，クラリスロマイシン，リネゾリドを記載しており[12]，筆者の施設でも投与を試みることがある．しかし，その有効性については定まった評価がされているわけではなく，WHOも「多剤耐性結核の通常の治療薬としては推奨しない」としている．

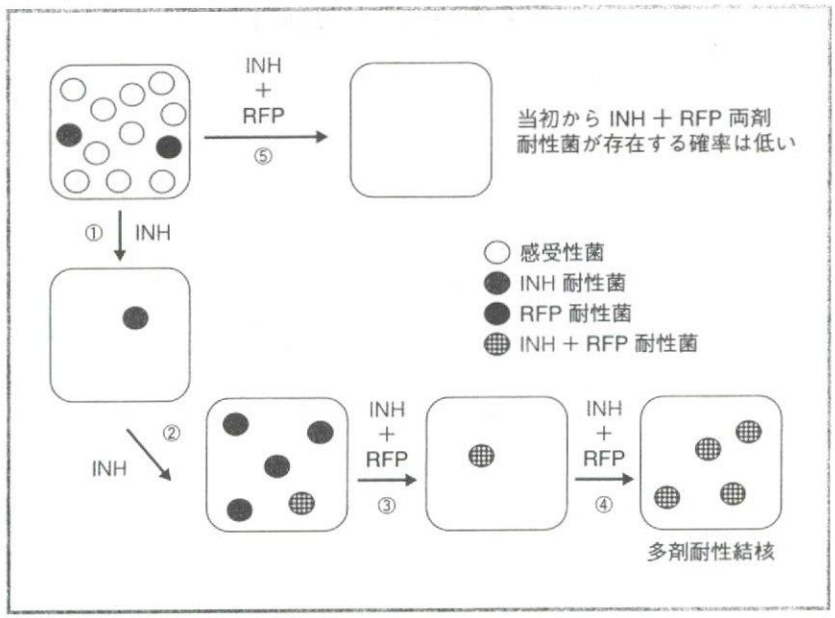
多剤耐性結核の病原性

結核菌の病原性を考えるとき，「感染させる力」と「発病させる力」に分けて考える必要がある．耐性結核菌は，野生株である感受性結核菌の突然変異により生じた菌であるため，従来はその病原性は弱いと考えられてきた．Riley らは，患者病室からの空気によるモルモットへの感染実験により，薬剤感受性結核患者では耐性結核患者に比して4倍から8倍感染性が高いことを示した[13]．また Cohn らは，INH 耐性菌ではカタラーゼ活性を欠き，動物実験で感受性菌より増殖が劣ることを報告した[14]．耐性菌は感染もしにくく，病気も作りにくいことが示唆されたわけである．

しかし，Snider らは，INH / SM 耐性菌と感受性菌で接触者に対する感染率に差がなかったことを報告した[15]．そして，インパクトが大きかったのは，1990 年代前半にニューヨークで多くの集団感染の原因となった多剤耐性結核菌株“strain W”の報告である[16]．この菌による結核患者の多くは HIV 感染者であったが死亡率は 80％ にのぼり，多剤耐性結核菌は弱い菌であるというそれまでのドグマを一変させた．この報告の後，多剤耐性結核菌の強い病原性を示唆する報告が次々となされている．Narvskaya らは，多剤耐性結核菌による院内集団感染事例を報告しており，発病者 19 人はすべて HIV 陰性であった[17]．筆者らは，感受性結核治療中の患者への再感染発病を含む，多剤耐性結核による院内集団感染事例を報告している[18]．

これらの報告からは，感染にしろ発病にしろ，決して多剤耐性結核菌の病原性は感受性菌に比して劣らないことがうかがえる．ただし，病状の進行もほとんどみられず接触者にも感染者を見いだせない，おそらく病原性が弱いと考えられる多剤耐性結核患者がいることも明らかである．おのおのの strain により病原性に差があると考えられるが，それを *in vitro* で検出する方法はない．重要なことは，病原性の強い多剤耐性結核菌が存在することを認識したうえで，結核対策を考えていくことであろう．

図1　多剤耐性結核までの経過例



多剤耐性結核をつくらないために

はじめに述べたように，耐性結核は医療従事者側の不適切な治療や，患者側のコンプライアンス不良などによる Man－made disease である．耐性結核をつくらないための原則は，① 結核の治療は多剤併用で行う，② 治療経過が思わしくないときに決して薬剤を単剤で追加しない，ことに尽きる．また，その地域での耐性率を考慮に入れておくことも必要であり，例えば INH 初回耐性率が 4.4％ にものぼる我が国[3] では，INH＋RFP の2剤による治療は RFP の単剤治療となる可能性が高いため行うべきではない．重症度，排菌の有無にかかわらず，すべての結核に対して，可能な限りピラジナミド（PZA）を含む4剤による標準化学療法を行うべきである．

筆者の経験した教訓的な症例を紹介する．症例は 70 歳代の女性で，湿性咳嗽で受診，胸部X線で肺結核が疑われたが喀痰で排菌が証明されないため，とりあえず INH 単剤による治療が開始された．自覚症状，画像所見が改善したためそのまま治療が継続されたが，その後再び悪化傾向となり，同時に喀痰検査で結核菌を認めたため慌てて RFP が追加された．再び改善を認めたが，2剤による治療を継続し

ていたところ再度悪化し，その際の喀痰培養菌は INH，RFP 耐性となっていた．この経過を図示すると図1のようになる．すなわち，当初は INH・RFP 両剤耐性菌は存在しないが，INH 単剤治療により一時的に菌量減少するが（①），その後 INH 耐性菌のみが選択され増殖し，その中に RFP にも耐性を獲得した多剤耐性菌が出現する（②）．その後 RFP が追加されると再び菌量減少するが（③），その後多剤耐性菌のみが増殖してしまう（④）．当初から多剤併用治療を行っていれば多剤耐性菌を誘導することなく治癒に至っていたわけである（⑤）．

このように「とりあえず INH のみを投与してみる」という治療が，最も行ってはならないことであり，成書でも結核疑診例にこのような単剤による診断的治療を勧めているものがあるが厳に慎むべきである．単剤治療が正当化されるのは，菌量が極めて少ないと考えられる化学予防の場合のみであるが，その場合でも活動性結核を慎重に除外診断しておくことの重要性が理解できよう．

露口一成

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Molecular Analysis of RANKL-Independent Cell Fusion of Osteoclast-Like Cells Induced by TNF-α, Lipopolysaccharide, or Peptidoglycan

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Abstract Focusing on the final step of osteoclastogenesis, we studied cell fusion from tartrate-resistant acid phosphatase (TRAP)-positive mononuclear cells into multinuclear cells. TRAP-positive mononuclear cells before generation of multinuclear cells by cell fusion were differentiated from RAW264.7 cells by treatment with receptor activator of nuclear factor kappa B ligand (RANKL), and then the cells were treated with lipopolysaccharide (LPS), followed by culturing for further 12 h. LPS-induced cell fusion even in the absence of RANKL. Similarly, tumor necrosis factor (TNF)-α and peptidoglycan (PGN) induced cell fusion, but M-CSF did not. The cell fusion induced by RANKL, TNF-α, and LPS was specifically blocked by osteoprotegerin (OPG), anti-TNF-α antibody, and polymyxin B, respectively. LPS- and PGN-induced cell fusion was partly inhibited by anti-TNF-α antibody but not by OPG. When TRAP-positive mononuclear cells fused to yield multinuclear cells, phosphorylation of Akt, Src, extracellular signal-regulated kinase (ERK), p38MAPK (p38), and c-Jun NH2-terminal kinase (JNK) was observed. The specific chemical inhibitors LY294002 (PI3K), PP2 (Src), U0126 (MAPK-ERK kinase (MEK)/ERK), and SP600125 (JNK) effectively suppressed cell fusion, although SB203580 (p38) did not. mRNA of nuclear factor of activated T-cells c1 (NFATc1) and dendritic cell-specific transmembrane protein (DC-STAMP) during the cell fusion was quantified, however, there was no obvious difference among the TRAP-positive mononuclear cells treated with or without M-CSF, RANKL, TNF-α, LPS, or PGN. Collectively, RANKL, TNF-α, LPS, and PGN induced cell fusion of osteoclasts through their own receptors. Subsequent activation of signaling pathways involving PI3K, Src, ERK, and JNK molecules was required for the cell fusion. Although DC-STAMP is considered to be a requisite for cell fusion of osteoclasts, cell fusion-inducing factors other than DC-STAMP might be necessary for the cell fusion. J. Cell. Biochem. 9999: 1–13, 2006.

Key words: TNF-α; lipopolysaccharide; peptidoglycan; cell fusion; osteoclasts

Abbreviations used: Akt, PKB, protein kinase B; c-Fos, cellular homolog of v-fos; c-Src, cellular homolog of v-src; DC-STAMP, dendritic cell-specific transmembrane protein; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; HRP; horse radish peroxidase; IL-1β, interluekine-1β; IL-6, interluekine-6; JNK, c-Jun NH2-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK-ERK kinase; MyD88, myeloid differentiation factor 88; NFATc1, nuclear factor of activated T-cells c1; NF-κB, nuclear factor kappa B; OPG, osteoprotegrin ligand; P-, phosphorylated; PBS, phosphate-buffered saline; PGN, peptidoglycan; p38, p38MAPK; p65, 65 kD subunit of NF-κB (RelA); RANKL, receptor activator of nuclear factor kappa B ligand; SDS, sodium dodecyl sulfate; TBST, 150 mM NaCl and 0.1% Tween-20 in 25 mM Tris/HCl, pH 7.6; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; TRAP, tartrate-resistant acid phosphatase;

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Bone is continuously remodeled by bone formation and resorption, and the cooperative bone metabolism is precisely regulated to maintain homeostasis. Osteoclasts, which are responsible for bone resorption in bone metabolism, are multinucleated cells originating from hematopoietic precursor cells of the monocyte/macrophage lineage. One of the key factors for osteoclastogenesis, which is induced by osteoblasts, is receptor activator of nuclear factor kappa B ligand (RANKL), a member of the tumor necrosis factor (TNF) family [Anderson et al., 1997; Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998]. RANKL was found to be expressed by T-cells [Wong et al., 1997; Josien et al., 1999; Kong et al., 1999] and B-cells [Li et al., 2000] as well as osteoblastic/stromal cells and to be essential for osteoclast differentiation. Receptor activator of nuclear factor kappa B (RANK), which is one of TNF receptor family members, is expressed in osteoclasts and their precursor cells as the receptor of RANKL [Josien et al., 1999; Li et al., 2000; Choi et al., 2001]. Downstream signaling through RANK is essential for osteoclastogenesis.

Osteoclasts are differentiated through multiple steps that include cell fusion at the latest step of differentiation, yielding multi-nuclear cells. Dendritic cell-specific transmembrane protein (DC-STAMP) was recently found to play a role in the cell fusion of osteoclasts, which seems to support the activity of bone resorption by osteoclasts [Kukita et al., 2004; Yagi et al., 2005]; however, the factors involved in and the precise mechanism of the cell fusion are unknown.

Deviation from the normal conditions of bone resorption results in bone diseases such as osteopetrosis, osteoporosis, and bone resorptive infectious disease. Periodontal disease is the most frequent bone resorptive infectious disease and is thought to be caused mainly by infection with Gram-negative bacteria. Such an infectious and pathological condition induces inflammation, resulting in bone resorption. A major bacterial factor for inflammation has been believed to be lipopolysaccharide (LPS), a main component of the cell surface of Gram-negative bacteria. LPS has the ability to induce proinflammatory cytokines such as TNF-α, interluekine-1β (IL-1β), and interluekine-6 (IL-6) in various kinds of cells [Wang and Ohura, 2002]. Bacterial components such as LPS or various cytokines elicited in infectious lesions may modulate physiological osteoclastogenesis, leading to a pathological bone resorptive condition.

In the present study, we investigated what bacterial components or cytokines affect osteoclastogenesis at the stage of cell fusion, what receptor molecules are involved in the cell fusion, and what signaling pathways are necessary for the cell fusion.

MATERIALS AND METHODS

Reagents

Anti-nuclear factor of activated T-cells c1 (NFATc1) (7A6), anti-phospho extracellular signal-regulated kinase (ERK) (E-4), anti-TRAF6 (H-274) and anti-cellular homolog of v-src (c-Src) were purchased from Santa Cruz (Santa Cruz, CA). TNF-α, anti-TNF-α, osteoprotegrin ligand (OPG), and transforming growth factor-β (TGF-β) were from R&D Systems Inc. (Mineapolis, MN). Anti-ERK, anti-p38 MAPK (p38) (5F11), anti-phospho-p38 (28B10), anti-Akt, anti-phospho-Akt, and anti-phospho-c-Jun NH2-terminal kinase (JNK) antibodies were from Cell Signaling Technology (Beverly, MA). RANKL was from Peprotech EC Ltd (London, United Kingdom). LY294002, PD169316, SB203580, SP600125, PP2, U0126, and PD98059 were purchased from Calbiochem Corp. (La Jolla, CA). M-CSF was from Kyowa Hakko Kogyo Co., Ltd (Tokyo, Japan). RANKL was from Peprotech (Rocky Hill, NJ). cDNA of mouse kidney was from Clontech. Minimal essential medium α modification (α-MEM) and all other reagents including LPS, PGN, IL-1β, and IL-6 were obtained from Sigma (St. Louis, MO).

Cell Culture

The murine monocytic cell line RAW264.7 (ATCC, Rockville, MD) was cultured in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C and maintained on uncoated plastic dishes of 9 cm in diameter in α-MEM containing 10% (v/v) heat-inactivated fetal calf serum (FCS) with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For subculture, the cells were resuspended with 10 ml of fresh medium by mild pipetting and transferred to a 15 ml conical tube. After sitting for 5 min, the upper 14.5 ml fraction was removed, and aggregated cells were collected from the bottom and seeded into

a fresh dish containing 12 ml of fresh medium. For osteoclastogenesis experiments, the indicated number of cells was seeded on tissue culture plates in the presence or absence of RANKL and chemical reagents.

Tartrate-Resistant Acid Phosphatase (TRAP) Staining

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for more than 60 min. The cells were then treated with 0.2% Triton X-100 in PBS at room temperature for 5 min, followed by rinsing twice with PBS. Finally, the fixed cells were stained with 0.01% naphthol AS-MX phosphate (Sigma) and 0.05% fast red violet LB salt (Sigma) in the presence of 50 mM sodium tartrate and 90 mM sodium acetate (pH 5.0) for 15–60 min and then rinsed twice with PBS.

Measurement of TRAP Intensity

Following TRAP staining, the plates were scanned by a transparent light scanner, and the red color image was extracted from the scanned image using the Photoshop (Adobe Systems Inc., San Jose, CA) computer program. The intensity of the red color image was measured using National Institutes of Health (NIH) Image computer program and is represented as TRAP intensity in this paper.

Cell Proliferation Assays

Cell proliferation was measured using a Cell Counting Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Similar to the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide] assay, this kit measures intracellular mitochondrial dehydrogenase activity in living but not in dead cells by forming water-soluble formazan dye with the tetrazolium compound WST-1, a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

Immunoblotting

Cells were seeded at 5×10^6 cells/well on a 24-well plate and kept in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C for 4 h. The cells were stimulated with RANKL or/and other chemical reagents and incubated for the indicated time. After incubation, the cells in the 24-well plate were rinsed twice with ice-cold PBS, followed by addition of 50 μl of sodium dodecyl sulfate (SDS)-sample buffer [1% SDS, 100 mM dithiothreitol (DTT), 10% glycerol, 0.0025% bromophenol blue, 62.5 mM Tris/HCl, and pH 6.8] containing 1 mM sodium orthovanadate (protein tyrosine phosphatase inhibitor), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1μg/ml chymostatin, and 1 μg/ml leupeptin. The whole cell lysate was then treated in ice-cold water by sonication (two 15-s pulses with a 60-s interval) (Bioruptor UDC-200T, Cosmo Bio, Tokyo, Japan). After boiling for 5 min, 4–7 μl of the lysate (20 μg of protein) was subjected to SDS- polyacrylamide gel electrophoresis (PAGE), and the proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in 5% skim milk, 25 mM Tris/HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20 (TBST) at room temperature for 1 h, washed twice with TBST for 5 min, and then incubated with an antibody at 2000–4000 × dilution in TBST at 4°C for 16 h. The membrane was washed three times with TBST for 10 min, incubated with a horse radish peroxidase (HRP)-conjugated second antibody at 4000–8000 × dilution in TBST at room temperature for 1 h, washed vigorously five times for 10 min, and subjected to chemiluminescence (ECL-plus, Amersham Pharmacia Biotech, Piscataway, NJ) to visualize HRP. In some experiments for reprobing, the membrane was stripped of antibody with Reblot Plus Strong (Chemicon, CA) according to the manufacturer's instructions.

Real-Time PCR

Total RNA was prepared using an RNeasy Mini Spin Column (Quiagen) according to the manufacturer's instructions, and the cDNA was reverse-transcribed by SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Using an M × 3005PTM Real-time PCR System and Brilliant SYBR Green QPCR Mastermix (Stratagene), the reverse-transcribed cDNA was amplified and quantifed with specific primers according to the manufacturer's instructions. The primer sequences used for amplification were as follows: mouse GAPDH, 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-CACATTGGGGGTAGGAACAC-3′; NF-ATc1, 5′-TCATCCTGTCCAACACCAAA-3′ and 5′-TCACCCTGGTGTTCTTCCTC-3′; DC-STA-MP, 5′-GGGCACCAGTATTTTCCTGA-3′ and 5′-TGGCAGGATCCAGTAAAA-

GG-3′; and TRAP, 5′-CAGCAGCCAAGGAG-GACTAC-3′ and 5′-ACATAGCCCACACCGT-TCTC-3′. The relative amounts of each mRNA were normalized by the GAPDH expression.

RESULTS

LPS-Mediated Inhibition in the Initial Step of Osteoclast Differentiation

LPS, a major component of the cell wall of Gram-negative bacteria, has been reported to strongly induce inflammation, which is thought to cause bone destruction. LPS induces bone resorption when administered in vivo; however, it suppresses osteoclast formation from bone marrow macrophages in vitro cell cultures. In order to determine what step of osteoclastogenesis is influenced by LPS, we examined whether LPS affected RANKL-induced osteoclastogenesis when it was present in RAW264.7 cell culture at different time periods after treatment with RANKL. In a previous study, we found that 1–3 μM U0126, a MAPK-ERK kinase (MEK)/ERK inhibitor, accelerated osteoclastogenesis of RAW264.7 cells in the presence of RANKL but that 10 μM of U0126 suppressed osteoclastogenesis [Hotokezaka et al., 2002]. In culture conditions with or without 2 μM U0126, cells were treated with LPS for different periods of 12 h during the total 48-h culture. In both culture conditions, LPS suppressed osteoclastogenesis when added for the first 12 h, and the

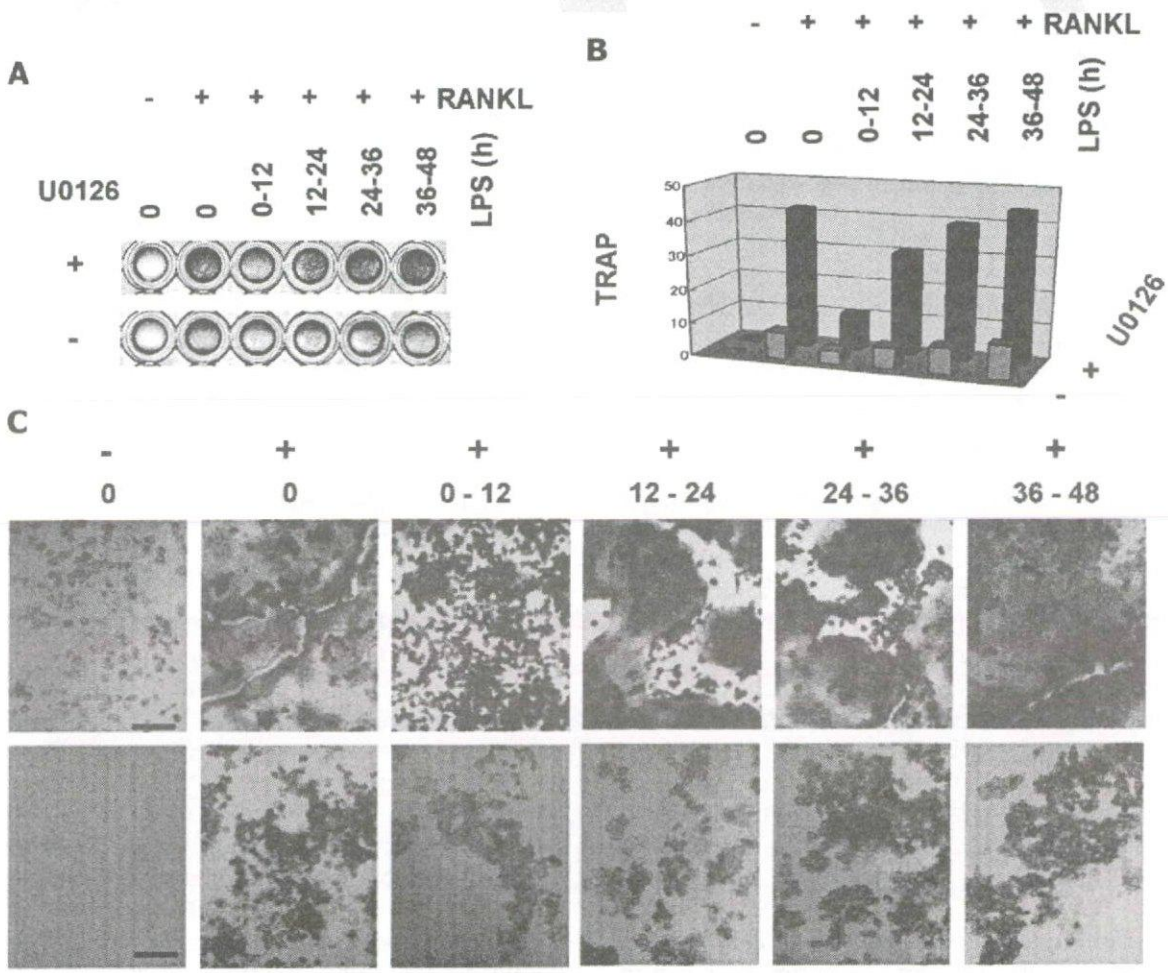


Fig. 1. Effect of LPS on differentiation of RAW264.7 cells into osteoclast-like cells. RAW264.7 cells in 250 μl of α-MEM were cultured in a 96-well tissue culture plate in the presence of 25 ng/ml RANKL with (upper; 16,000 cells) or without (lower; 8,000 cells) 2 μM U0126. The cells were stimulated with 25 ng/ml LPS during the indicated period in the absence of RANKL. TRAP activity was visualized by TRAP staining. **A:** culture plate scanned. **B:** measured TRAP intensity, the mean of three different determinations was plotted. **C:** cell shapes under an optical microscope. Bars indicate 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

suppression was markedly weaker when LPS was added at a later 12-h period (Fig. 1). Similar to the previous reports [Takami et al., 2002; Zou and Bar-Shavit, 2002; Zou et al., 2002; Hayashi et al., 2003], LPS inhibits osteoclastogenesis only in the early step of differentiation suggests that effects of cytokines or Toll-like receptor (TLR) ligand molecules such as LPS on osteoclastogenesis may vary among steps of differentiation.

Effects of Cytokines or TLR Ligands on the Latest Step of Osteoclastogenesis

In this study, we used an enhanced culture condition in which the cells were cultured in the presence of both RANKL and U0126 since TRAP-positive multinuclear cells were generated in the culture condition within 48 h (Fig. 1). Effects of various cytokines or TLR ligands on the latest step of osteoclastogenesis and fusion of TRAP-positive mononuclear cells were investigated. First, TRAP-positive mononuclear cells were generated from RAW264.7 cells by incubation with RANKL and U0126 for 36 h. The cells were then incubated in culture medium with various cytokines or TLR ligand molecules for 12 h in the absence of RANKL. Similar to the previous reports [Lam et al., 2000; Zou and Bar-Shavit, 2002; Zou et al., 2002], LPS, PGN, and TNF-α induced cell fusion as well as RANKL (Fig. 2). Although M-CSF failed to induce cell fusion, it increased the number of TRAP-positive mononuclear cells. IL-1β had no ability to induce cell fusion in this condition. In addition, we examined IL-6 (0.1–50 ng/ml), TGF-β (0.1–50 ng/ml), phorbol myristic acetate (PMA; 0.1–10 μM), concanavalin A (0.1–10 μg/ml), amphotericin B (AmB; 0.1–10 μg/ml), and oxydized low-density lipoprotein (LDL; 0.1–10 μg/ml). None of them induced cell fusion (data not shown) although PMA, AmB, and oxydized LDL are known as inducers of proinflammatory cytokines in macrophages [Stuart and Hamilton, 1980; Cleary et al., 1992; Pollaud-Cherion et al., 1998]. These results suggested that cell fusion in the latest step of osteoclastogenesis might be induced by treatment with RANKL, TNF-α, LPS, and PGN but not by treatment with others.

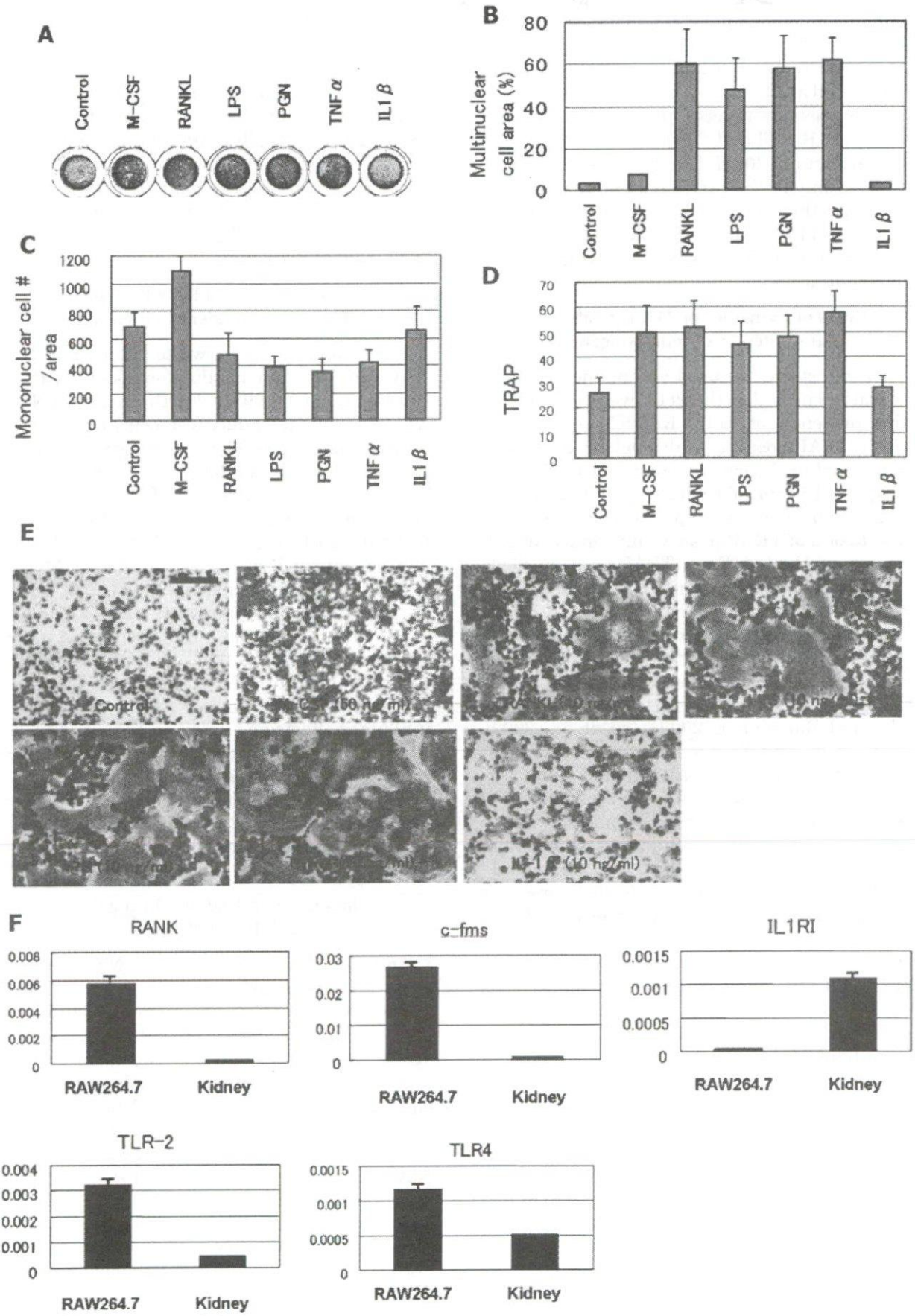
It is known that IL-1β-induced cell signaling is similar to that of LPS and PGN with respect to involvement of MyD88, an adaptor molecule of IL1R family members [Subramaniam et al., 2004]. We investigated whether the IL-1β receptor IL1RI was expressed in RAW264.7 cells. Real-time RT-PCR analysis revealed that IL1RI was not expressed in RAW264.7 cells, whereas RANK, c-fms, TLR2, and TLR4, receptors for RANKL, M-CSF, PGN, and LPS, respectively, were expressed in the cells (Fig. 2F). Absence of the IL-1β receptor in the cell line may account for the disability of IL-1β to induce cell fusion.

Ability of LPS and PGN to Induce TNF-α-Independent Cell Fusion

LPS and PGN are known to induce TNF-α in RAW264.7 and other cells [Remick et al., 1988; Gupta et al., 1995]. Furthermore, TNF-α was reported to induce osteoclastogenesis in macrophages exposed to permissive levels of RANKL [Lam et al., 2000]. In order to determine whether cytokines or TLR ligands directly or indirectly induced cell fusion, OPG—a neutralizing antibody against TNF-α (anti-TNF-α), and polymyxin B were used to block RANKL, TNF-α, and LPS, respectively (Fig. 3). OPG, anti-TNF-α, and polymyxin B (each 0.3 μg/ml) specifically inhibited the osteoclast cell fusion induced by RANKL, TNF-α, and LPS, respectively. Anti-TNF-α strongly inhibited TNF-α-induced cell fusion and also had some inhibitory effects at 1 μg/ml on LPS- and PGN-induced cell fusion (Fig. 3A). Anti-TNF-α (1 μg/ml) completely suppressed cell fusion induced by TNF-α (10 ng/ml) and the secretion of TNF-α induced by LPS or PGN was less than 1.2 ng/ml, suggesting that LPS and PGN had the ability to induce TNF-α-independent cell fusion (Fig. 3B).

Immunoblot Analysis During the Cell Fusion Process

An intracellular domain of RANK interacts with TNF receptor-associated factor (TRAF) 2 and TRAF 6 [Galibert et al., 1998; Wong et al., 1998, 1999; Darnay et al., 1999; Kim et al., 1999], which appear to be involved in the activation of downstream signaling molecules such as nuclear factor kappa B (NF-κB), Src [Horne et al., 1992; Lowe et al., 1993], phosphatidylinositol 3 (PI3), protein kinase B (PKB/Akt), and mitogen-activated protein kinases (MAPKs) including p38, ERK, and JNK [Xia et al., 1995; Wong et al., 1998, 1999; Matsumoto et al., 2000; Chang and Karin, 2001; Lee et al., 2002; Wei et al., 2002]. In order to study cell signaling during the cell fusion process, we determined the amounts of several


A
Control
M-CSF
RANKL
LPS
PGN
TNF α
IL1 β
B
Multinuclear cell area (%)
80
60
40
20
0
Control
M-CSF
RANKL
LPS
PGN
TNF α
IL1 β
C
Mononuclear cell # /area
1200
1000
800
600
400
200
0
Control
M-CSF
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70
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0.008
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TLR4
0.0015
0.001
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Fig. 2.

transcription factors and phosphorylation of various signaling molecules by immunoblot analysis. In our culture system, TRAP-positive mononuclear cells began to fuse at 8 h, and they were fully fused at 16 h after stimulation. As shown in Figure 2, RANKL, TNF-α, and LPS equally induced fusion of TRAP-positive mononuclear cells, but M-CSF did not. Phosphorylation of Akt, Src, ERK, JNK, and p38 was observed at 480 or 960 min in cells stimulated with RANKL, TNF-α, and LPS (Fig. 4). p38 showed the most prominent phosphorylation by stimulation with RANKL, TNF-α, and LPS throughout the incubation period. Continuous phosphorylation of Akt was observed in cells treated with M-CSF, in contrast to the transient phosphorylation in cells treated with RANKL, TNF-α, or LPS at 8 and 16 h. The continuous or transient phosphorylation of Akt may be involved in cell fate, growth, or fusion. Since phosphorylation of signaling molecules is thought to be involved in the subsequent cellular responses, we determined the importance of the signaling molecules in the cell fusion process by using specific inhibitory compounds. As expected, LY294002 (PI3K inhibitor), PP2 (Src inhibitor), U0126 (ERK inhibitor), and SP600125 (JNK inhibitor) efficiently inhibited the RANKL-induced cell fusion, but SB203580 (p38 inhibitor) had no inhibitory effect (Fig. 5). Another p38 inhibitor, PD169316, also had no effect (data not shown). In order to study whether p38 is involved in an activity of osteoclast, resorption pit formation was measured. However, resorption pit formation induced by RANKL, TNF-α, or LPS was not inhibited by the p38 inhibitor SB203580. The role of p38 during the cell fusion process in osteoclastogenesis remains unclear.

Real-Time PCR Analysis of Expression of NFATc1, DC-STAMP, and TRAP

It was recently found that DC-STAMP participates in the cell fusion of osteoclasts [Kukita et al., 2004; Yagi et al., 2005]. First, we determined levels of mRNA of NFATc1, DC-STAMP, and TRAP in cells treated with RANKL at several time points. The mRNA level of NAFTc1 increased at an earlier time point than did the mRNA levels of DC-STAMP and TRAP. DC-STAMP and TRAP mRNAs increased immediately before cell fusion (Fig. 6). We then investigated the expression of NFATc1, DC-STAMP, and TRAP in the latest step of osteoclastogenesis. RAW264.7 cells that had been treated with RANKL for 36 h were then treated with RANKL, M-CSF, LPS, or TNF-α for 6 h. At this time point, the cells began to fuse. Then the expression of NFATc1, DC-STAMP, and TRAP in the cells was determined by real-time PCR analysis. Expression of DC-STAMP in RANKL- or LPS-treated cells was not significantly different from that in M-CSF-treated cells or non-treated cells. The M-CSF-treated cells and non-treated cells showed no cell fusion, whereas RANKL-, LPS-, and TNF-α-treated cells showed cell fusion. We could not find any significant difference in expression of NFATc1, DC-STAMP, and TRAP between the fused cells and non-fused cells. These results suggest that an essential factor other than DC-STAMP may determine whether TRAP-positive mononuclear cells are fused or not.

DISCUSSION

In an in vitro culture system using osteoclast precursor cells purified from various tissues such as bone marrow, spleen, and liver, it is difficult to avoid contamination with other cell lineages such as T-cells and mesenchymal stromal cells. In this study, we used RAW264.7—a murine macrophage cell line that can differentiate into osteoclast-like cells in the presence of RANKL [Hsu et al., 1999]. There are some characteristic differences between RAW264.7 cells and macrophages; for example, RAW264.7 cells do not respond to IL-1β, and the osteoclast-like cells differentiated from RAW264.7 cells form smaller and shallower

Fig. 2. Effects of factors on cell fusion of osteoclasts. RAW264.7 cells (1.6×10^5) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 10% FCS and indicated factors. Concentrations used were 50 ng/ml for M-CSF, 10 ng/ml RANKL, 10 ng/ml LPS, 100 ng/ml PGN, 10 ng/ml TNF-α, and 50 ng/ml IL-1β. **A:** scanned culture plate. **B:** area % of multinuclear cells. **C:** number of mononuclear cells. **D:** TRAP intensity. **E:** cell shapes under an optical microscope. **F:** mRNA quantified by real-time RT-PCR. Total RNAs were prepared from RAW264.7 cells treated with RANKL and U0126 for 36 h and kidney cells of mice, and they were subjected to real-time RT-PCR using primers for RANK, c-fms, TLR4, and IL-1R type 1. Bar indicates 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

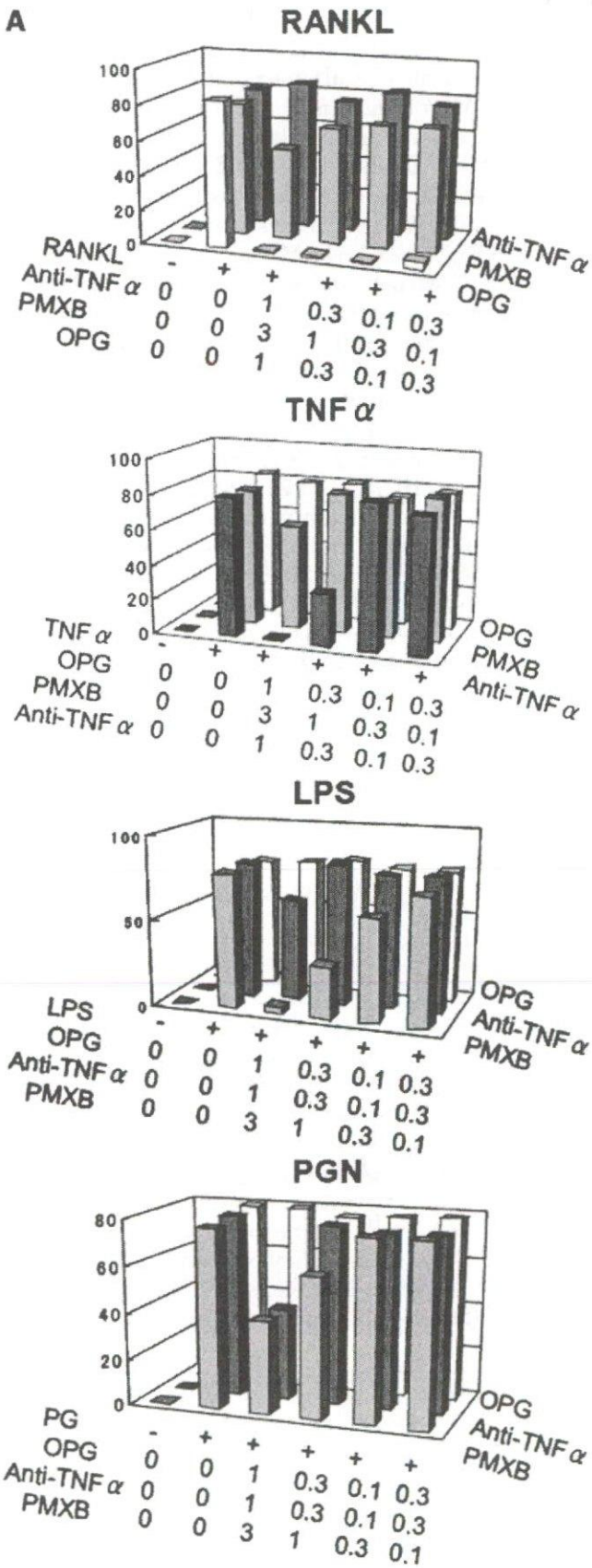


Fig. 3.

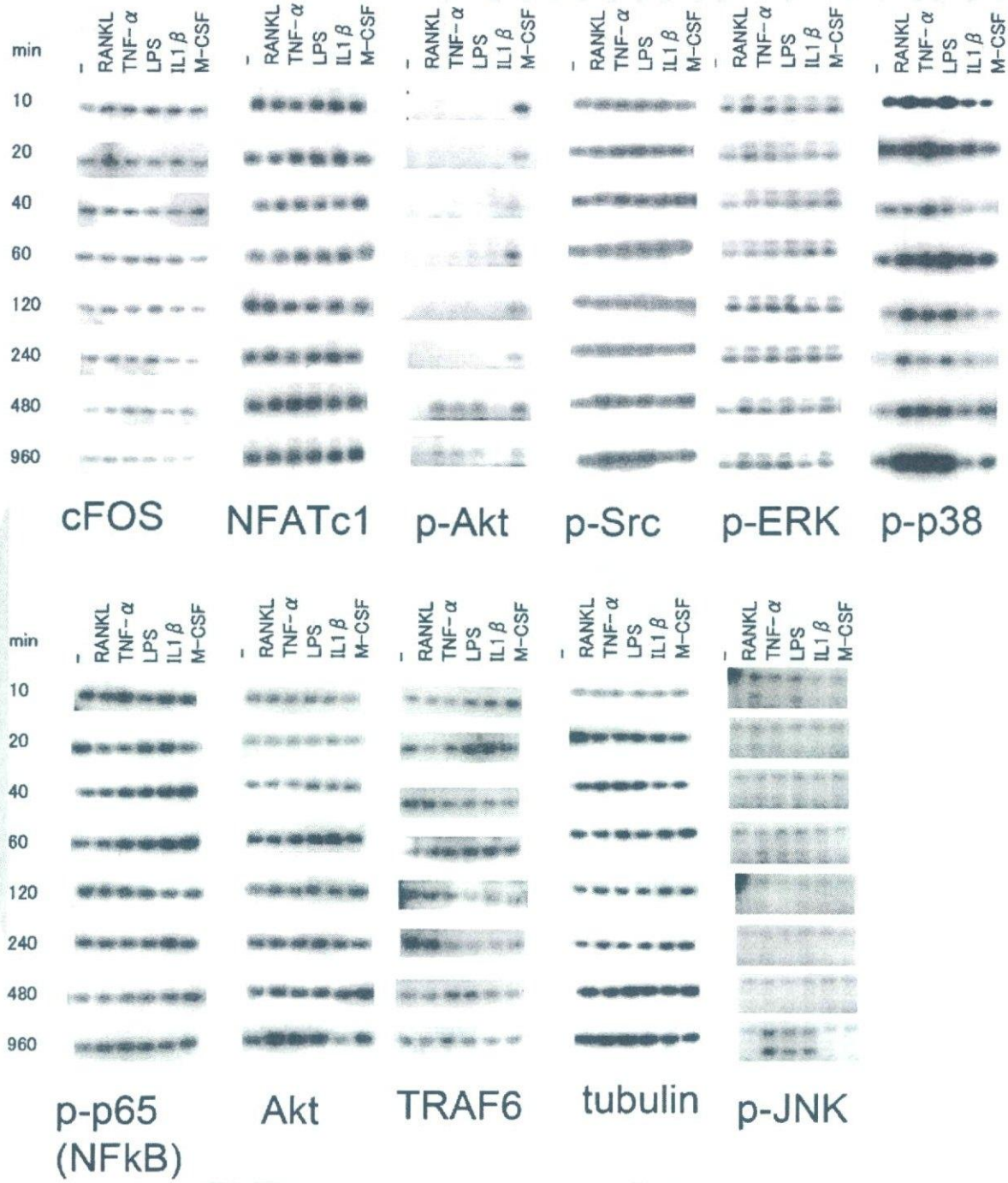
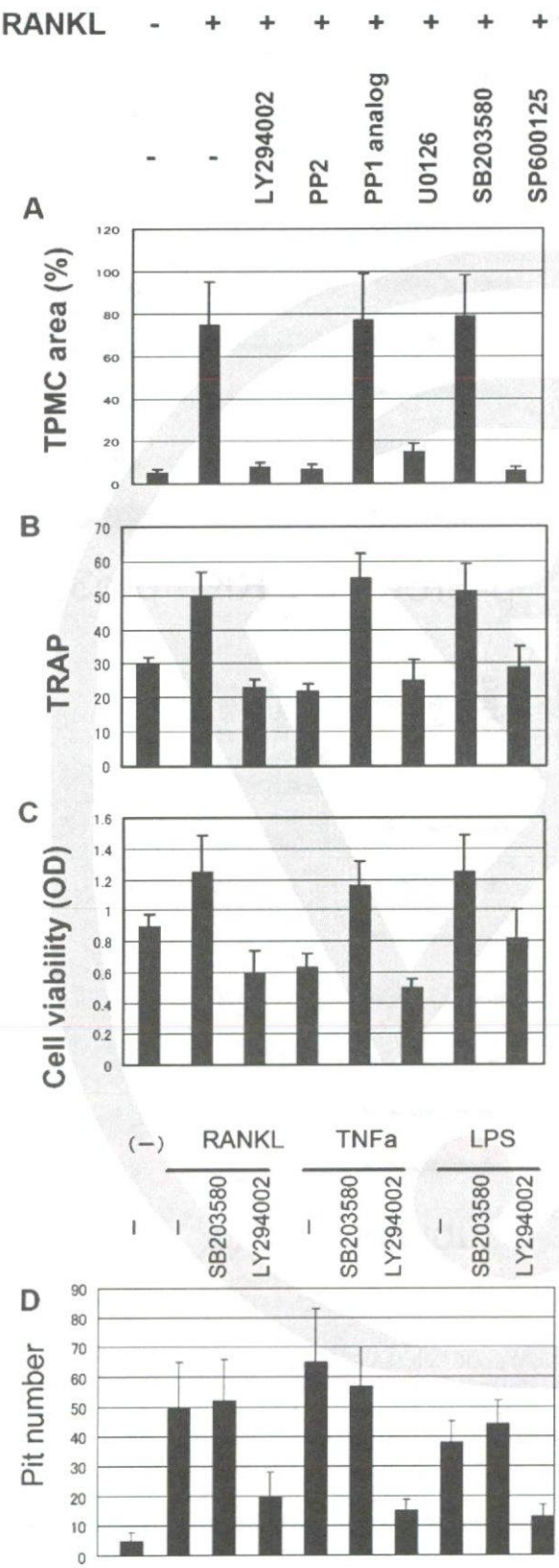


Fig. 4. Western blotting analysis during cell fusion process. The cells were treated with 25 ng/ml RANKL, 25 ng/ml TNF-α, 25 ng/ml LPS, or 100 ng/ml PGN for the indicated time and then subjected to Western blotting analysis probing with antibodies against c-Fos, NFATc1, phosphorylated (P-) Akt, P-Src, P-ERK, P-p38, P-p65 (RelA, 65 kD subunit of NF-κB), Akt, TRAF6, tubulin, and P-JNK.

Fig. 3. RANKL, TNF-α, LPS, and PGN induced cell fusion specifically through their receptors. RAW264.7 cells (1.6 × 105) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 10% FCS, indicated stimulation factors (RANKL, TNF-α, LPS, and PGN), and blocking reagents (OPG, PMXB, and Anti-TNF-α; indicated number μg/ml were used). The fused cell area was measured as percent of total plate area. Concentrations used were 10 ng/ml RANKL, 10 ng/ml TNF-α, 10 ng/ml LPS, and 100 ng/ml PGN (**A**). Amounts of TNF-α in the culture media stimulated with RANKL, LPS, and PGN were measured by ELISA (**B**). The mean of three different determinations was plotted.



resorption pits on dentin slices than do osteoclasts derived from bone marrow macrophages. However, the cell line is useful to analyze the detailed mechanisms of osteoclast differentiation.

In this study, we focused on the cell fusion step of osteoclastogenesis. Several investigators reported the later stage of osteoclastogenesis [Lam et al., 2000; Zou and Bar-Shavit, 2002; Zou et al., 2002]. However, this process is not characterized well since it is not easy to isolate cells at each step during the process of osteoclast differentiation. In this study, we used an enhanced culture condition for osteoclastogenesis of RAW264.7 cells. By culturing the cell line in the presence of RANKL and the MEK/ERK inhibitor U0126, the culture period for osteoclastogenesis was shortened to 48 h, less than half of the standard culture period, and almost all the cells are fused to one another at the end of the culture period. Therefore, the TRAP-positive mononuclear cells (preosteoclasts) prepared shortly before cell fusion in the enhanced culture condition appear to be almost homogeneous in terms of differentiation.

In the present study, LPS suppressed RANKL-induced osteoclast formation at the initial step; in contrast, at the latest step it induced cell fusion without RANKL, resulting in osteoclast formation. The suppressive effect of LPS on osteoclastogenesis, which was observed in the present study when LPS and RANKL were simultaneously added at the initial step, has previously been reported for bone marrow macrophages by Takami et al. [2002]. Moreover, it has been reported that LPS promotes the survival of mature osteoclasts via TLR4 [Itoh et al., 2003]. Taken together, the results indicate that the effects of LPS on osteoclastogenesis vary among steps of osteoclast differentiation. In this context, it is notable

Fig. 5. Effects of chemical inhibitors of signal transduction on cell fusion and pit formation. RAW264.7 cells (1.6×10^5) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, TNF-α, and 10 of chemical inhibitors, and the cells were cultured for a further 12 h. The fused cell area was measured as percent of total plate area (**A**). The TRAP intensity and cell viability were also measured (**B** and **C**). **D:** Pit formation was measured on a plate coated with calcium phosphate (BD BioCoat Osteologic Bone Cell Culture System, Nippon BD, Tokyo, Japan) stimulated with 25 ng/ml RANKL, TNF-α, or LPS in the presence or absence of 10 μM SB203580 or LY294002.

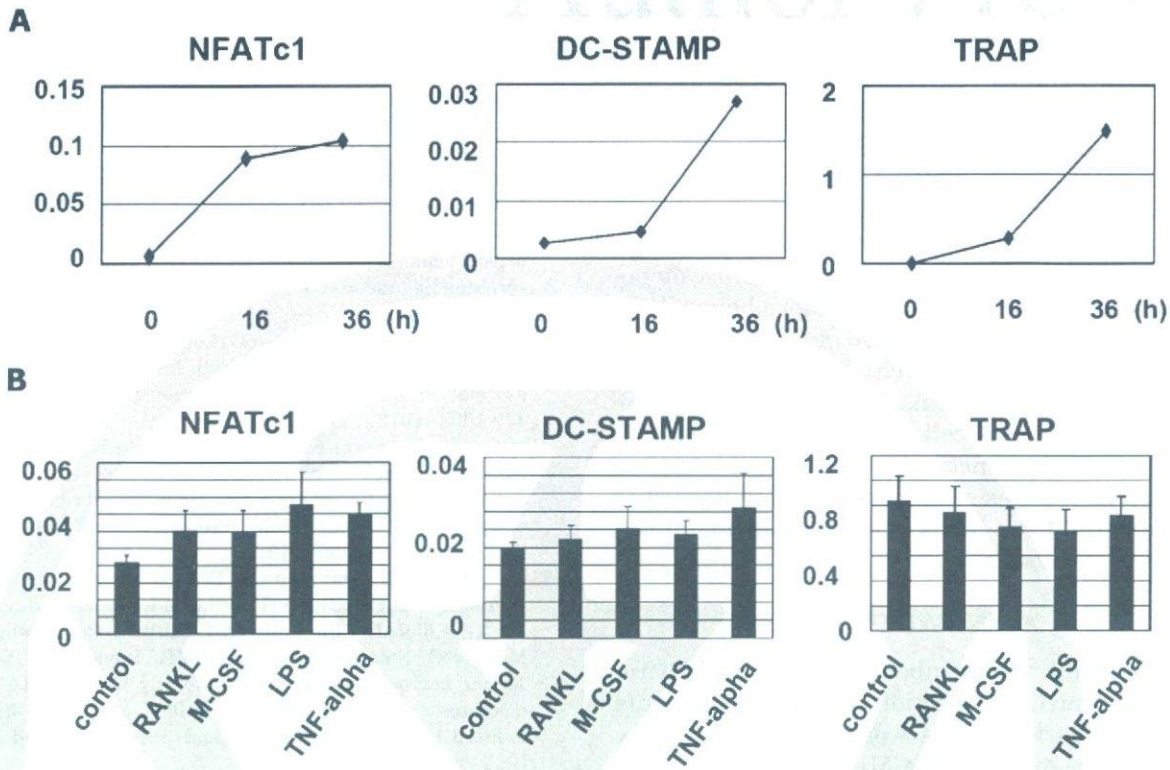


Fig. 6. Real-time PCR for NFATc1, DC-STAMP, and TRAP. RAW264.7 cells (1.6×10^5) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate (**A**). Then the medium was replaced with 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, or TNF-α, and the cells were cultured for further 6 h (**B**), followed by RNA preparation and real-time RT-PCR. Each value was normalized by that of GAPDH. The mean of three different determinations was plotted.

that LPS induces bone resorption when injected into bone surfaces of mice [Umezu et al., 1989]. Since osteoclast precursor cells of various steps of differentiation exist together in an in vivo condition, osteoclast precursor cells of the pre-fusion step around bones might differentiate into mature osteoclasts in response to LPS, resulting in bone resorption.

Cell fusion is seen in muscle, nerves, bone in their development, and in the liver in its repair and regeneration [Ogle et al., 2005]. TRAP-positive multinuclear osteoclasts appear in bone. TRAP-positive mononuclear cells fuse to one another because the cell fusion increases cell size and enables the cells to resorb bone to a larger extent [Vignery, 2005]. This explanation is supported by the finding that a DC-STAMP knockout mouse in which TRAP-positive mononuclear cells do not fuse to one another shows an increase in bone density due to a decrease in bone resorption by osteoclasts [Yagi et al., 2005]. Osteoclasts seem to have the same origin in cell lineage as that of macrophages. Macrophages sometimes fuse with one another during infection and tissue repair. The resulting multinucleated macrophages effectively phagocytise pathogens and repair tissues. The mechanism by which macrophages repair tissues seems to be similar to bone resorption of osteoclasts. In this study, inflammatory factors such as TNF-α, LPS, and PGN induced cell fusion, which may be one of mechanisms to repair the inflammatory circumstance of bone, although the inflammation results in an unfavorable bone loss.

Cell fusion-inducing factors include several groups. One is known as fusogens that can directly induce cell fusion. And other groups that include receptors, signaling proteins, transcription factors, and proteins organizing cytoskeleton and membrane, indirectly induce cell fusion [Ogle et al., 2005]. Although EFF-1 of *Caenorhabditis elegans* might be the only one fusogen that have been found in higher eukaryotes so far as we know [Kontani and Rothman, 2005], no fusogens have been found in mammalian cells. Some molecules such as meltrin-α [Harris et al., 1997; Inoue et al.,

1998], CD47, MFR (also reported as SIRP, SHPS-1, BIT, and MyD-1) [Vignery, 2005], and DC-STAMP have been suggested as cell fusion-inducing factors in osteoclasts. In the bones of mice lacking DC-STAMP multinuclear osteoclasts were completely absent, although development of mononuclear osteoclasts was normal. The DC-STAMP-deficient mice suffer from mild osteopetrosis probably because mononuclear osteoclasts can still resorb bones [Yagi et al., 2005]. The expression of DC-STAMP was not significantly different between fused and non-fused cells in this study, suggesting the presence of other cell fusion-inducing factor(s) that directly function at the latest stage of osteoclastogenesis. Further study is needed to understand the molecular mechanisms of cell fusion of osteoclasts.

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