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 $5 \times 10^6$ 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′-ACCCAGAAGACTGTG-GATGG-3′ and 5′-CACATTGGGGGTAGGAA-CAC-3′; NF-ATc1, 5′-TCATCCTGTCCAACA-CCAAA-3′ and 5′-TCACCCTGGTGTTCTTCC-TC-3′; DC-STA-MP, 5′-GGGCACCAGTATTTT-CCTGA-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

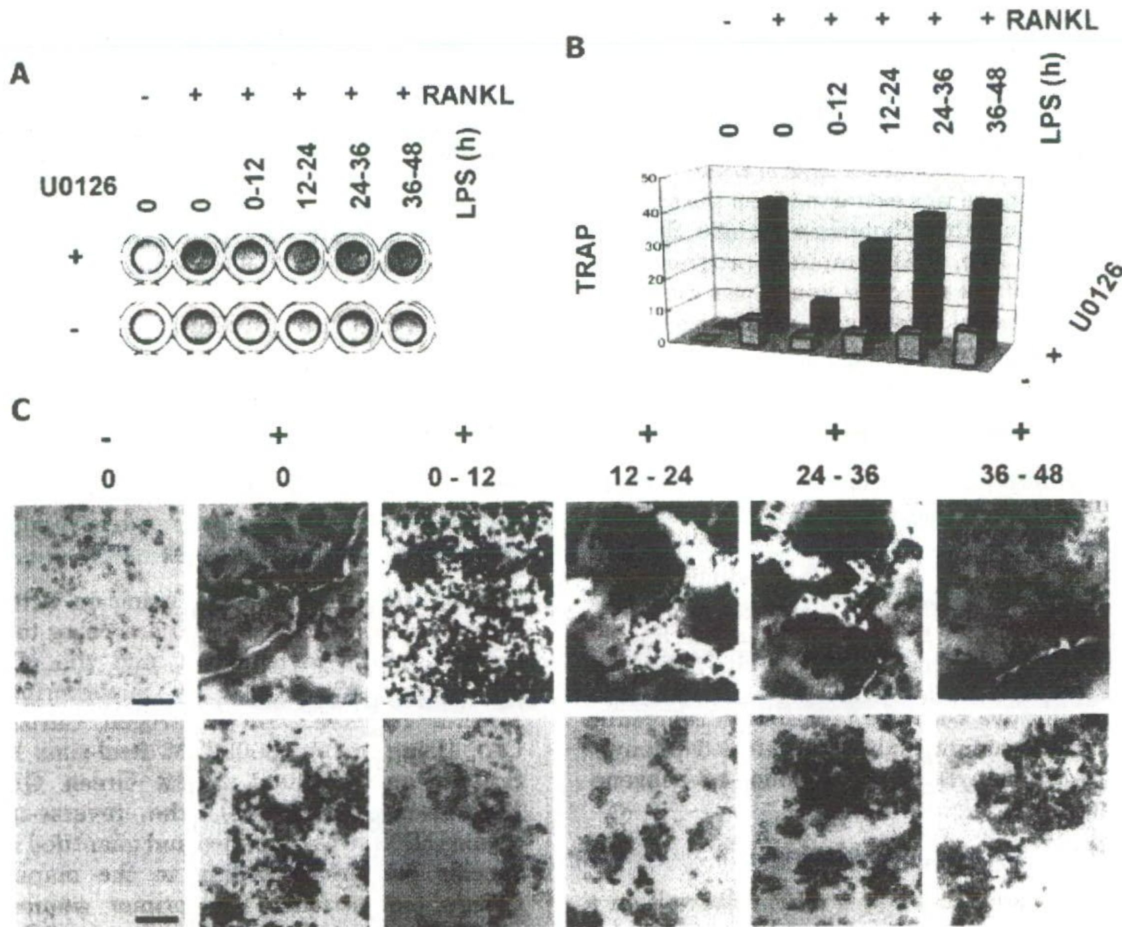



**Fig. 1.** Effect of LPS on differentiation of RAW264.7 cells into osteoclast-like cells. RAW264.7 cells in 250 μl of α-MEM were cultured in a 96-well tissue culture plate in the presence of 25 ng/ml RANKL with (upper; 16,000 cells) or without (lower; 8,000 cells) 2 μM U0126. The cells were stimulated 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).

**Fig. 2.** Effects of factors on cell fusion of osteoclasts. RAW264.7 cells ($1.6 \times 10^5$) 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.]

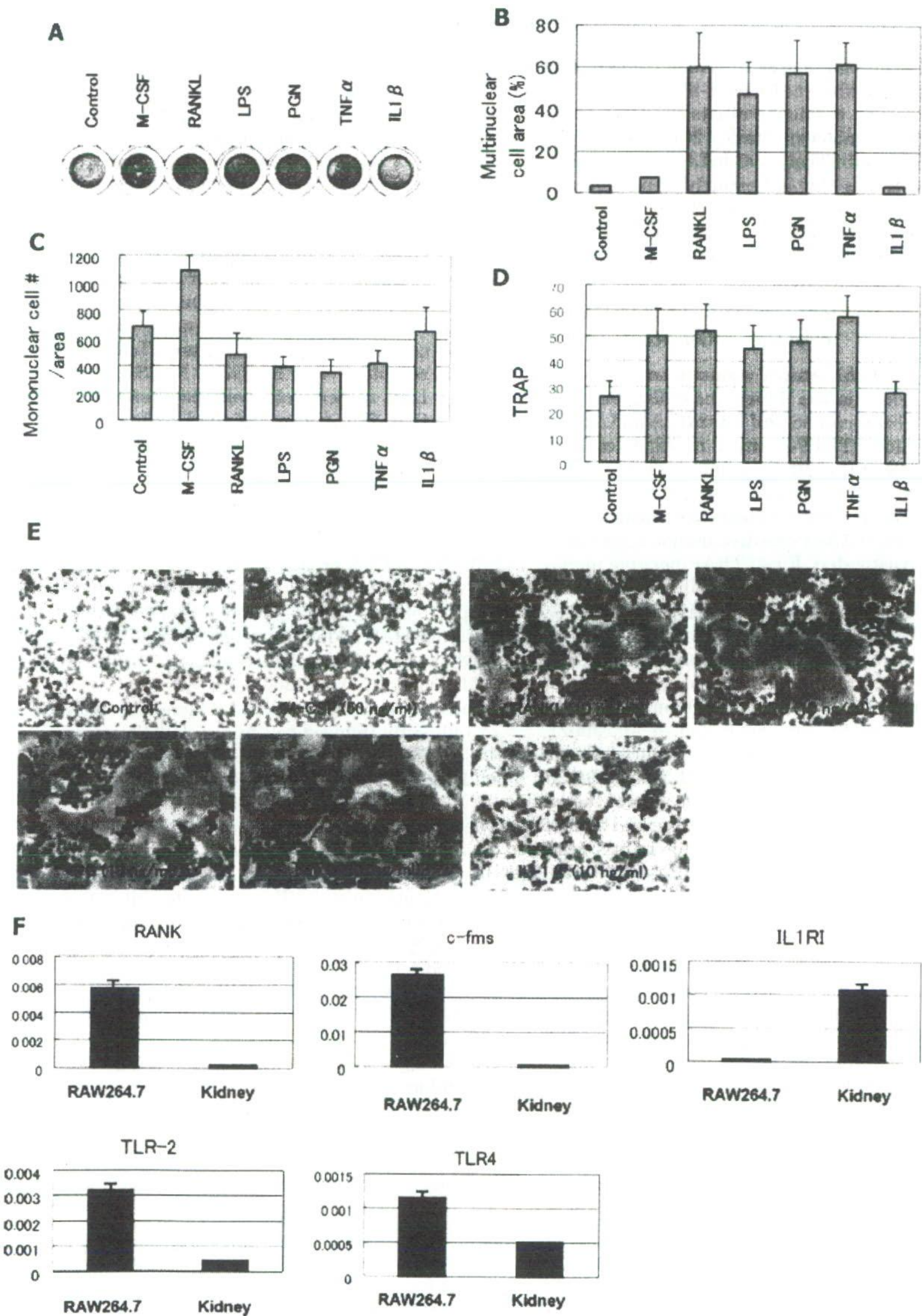



Fig. 2.

### Immunoblot Analysis During the Cell Fusion Process

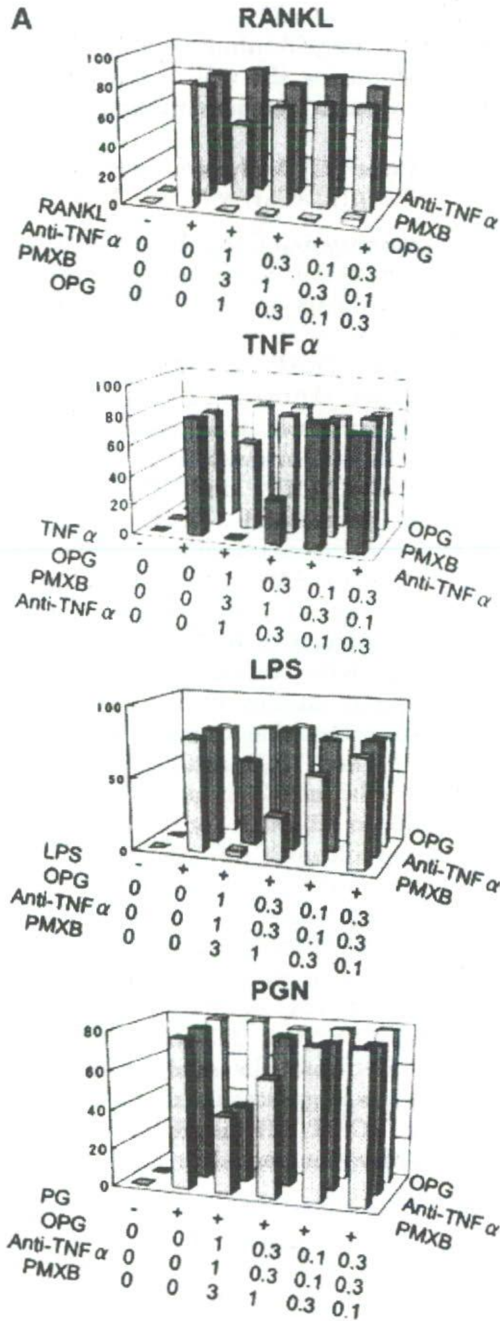
An intracellular domain of RANK interacts with TNF receptor-associated factor (TRAF) 2 and TRAF 6 [Galibert et al., 1998; Wong et al., 1998, 1999; Darnay et al., 1999; Kim et al., 1999], which appear to be involved in the activation of downstream signaling molecules such as nuclear factor kappa B (NF-κB), 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 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



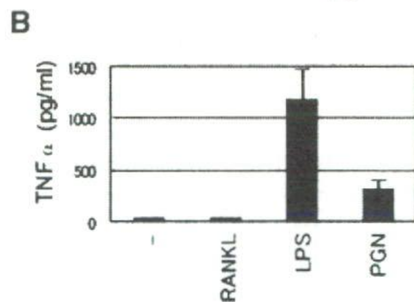




Fig. 3. RANKL, TNF-α, LPS, and PGN induced cell fusion specifically through their receptors. RAW264.7 cells (1.6 × 105) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 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.

the osteoclast-like cells differentiated from RAW264.7 cells form smaller and shallower 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

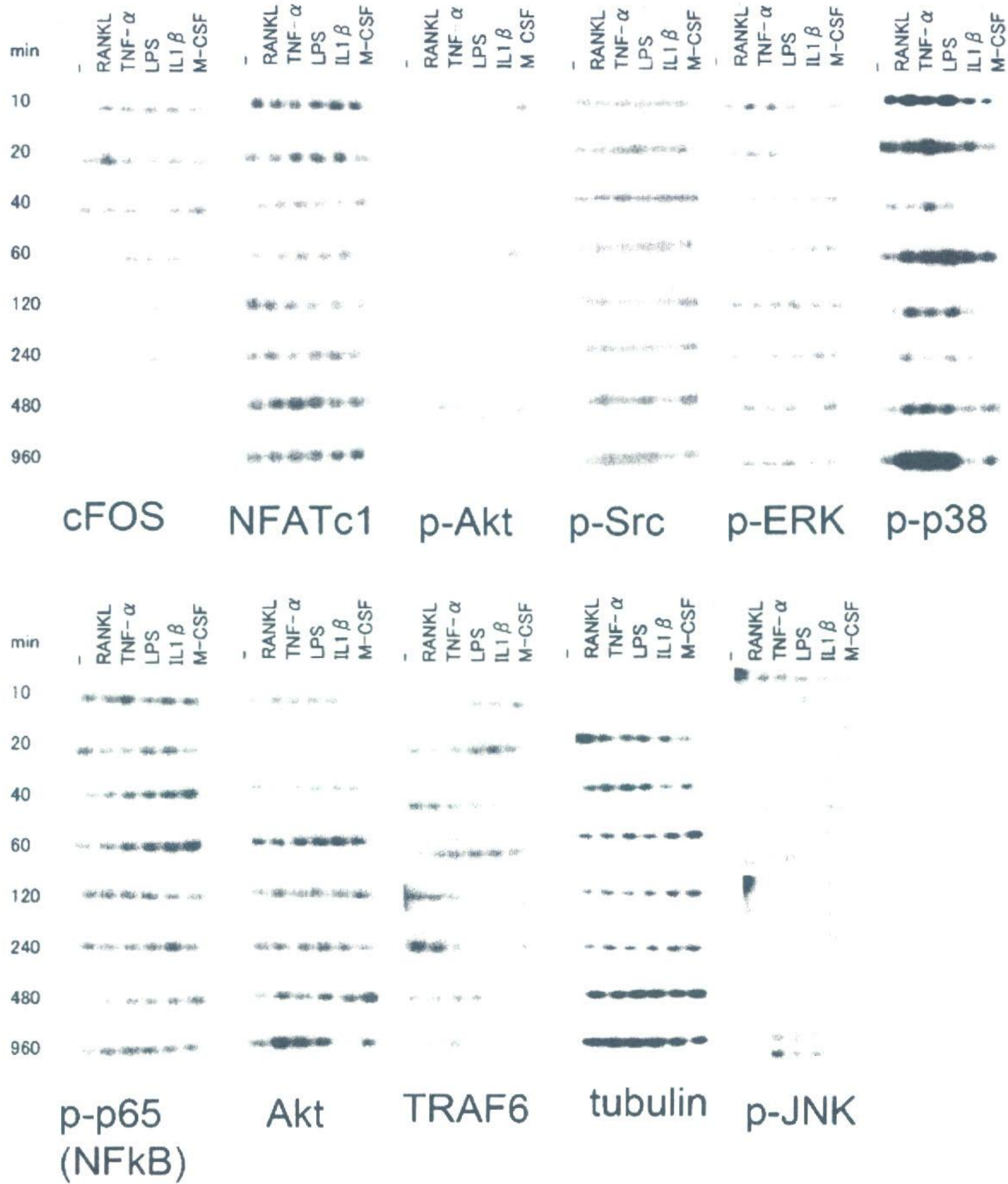



**Fig. 4.** Western blotting analysis during cell fusion process. The cells were treated with 25 ng/ml RANKL, 25 ng/ml TNF-α, 25 ng/ml LPS, or 100 ng/ml PGN for the indicated time and then subjected to Western blotting analysis probing with antibodies against c-Fos, NFATc1, phosphorylated (P-) Akt, P-Src,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

osteoclastogenesis vary among steps of osteoclast differentiation. In this context, it is notable 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.

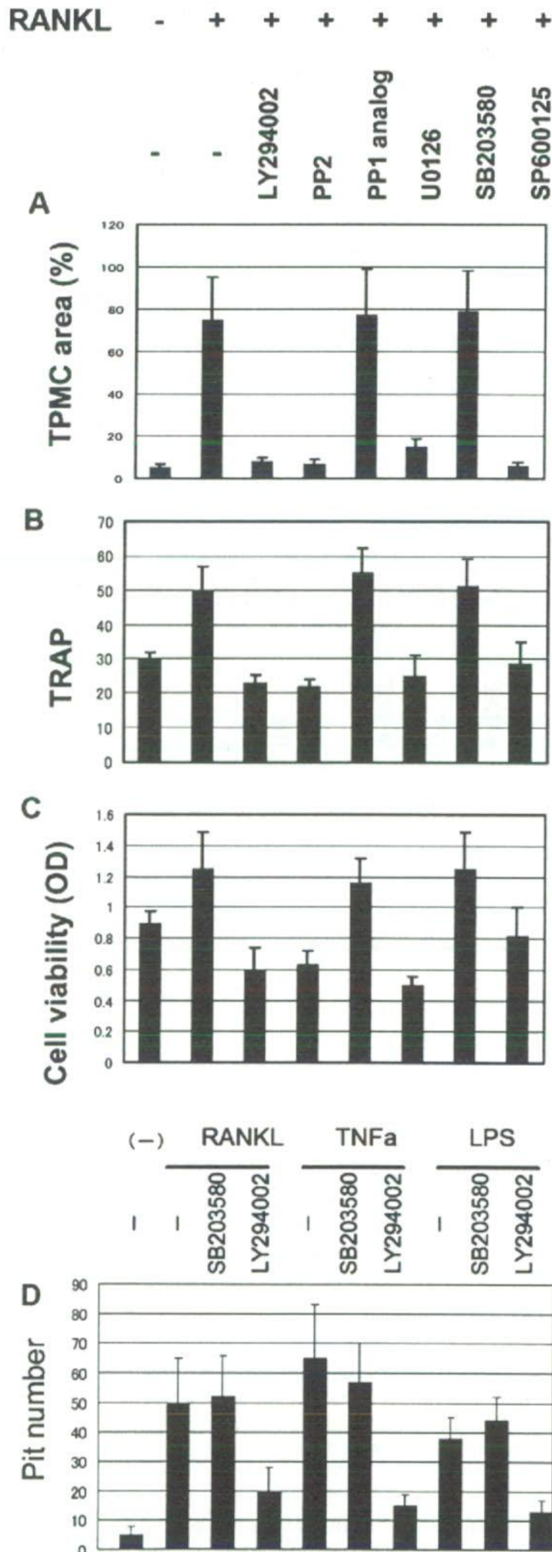



Fig. 5. Effects of chemical inhibitors of signal transduction on cell fusion and pit formation. RAW264.7 cells ($1.6 \times 10^5$) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of 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.

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,

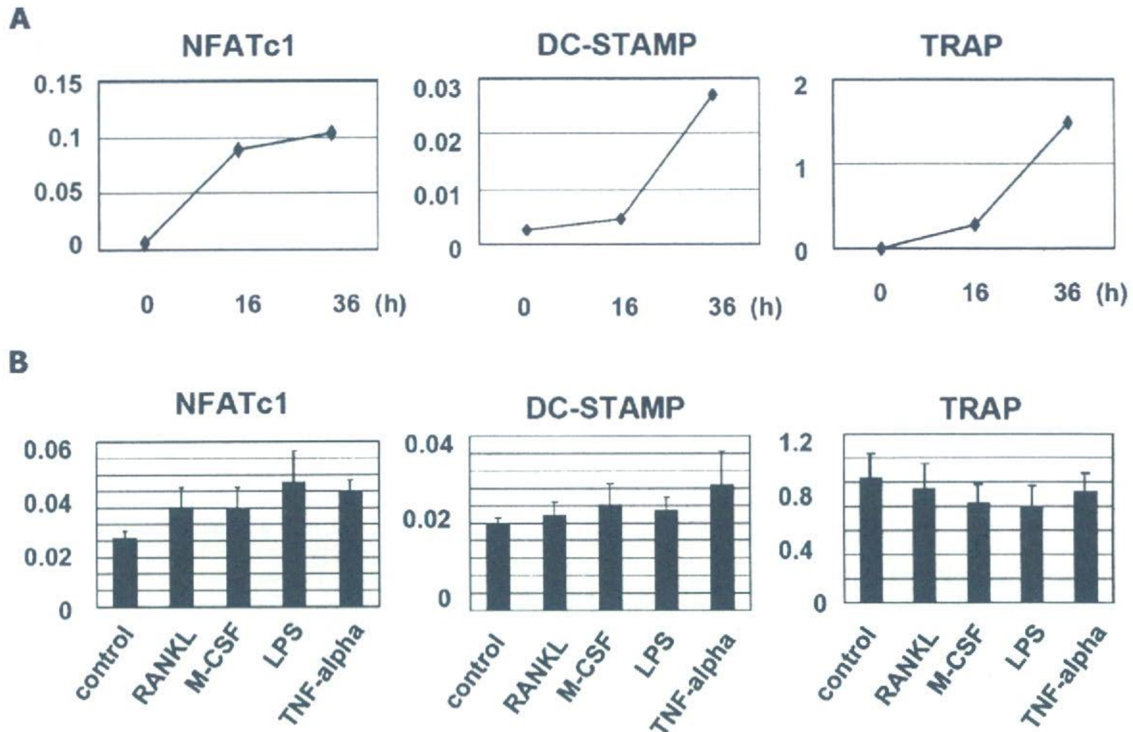



**Fig. 6.** Real-time PCR for NFATc1, DC-STAMP, and TRAP. RAW264.7 cells ($1.6 \times 10^5$) were cultured for 36 h in 250 µl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 µM U0126 in a 96-well tissue culture plate (**A**). Then the medium was replaced with 250 µl of α-MEM containing 10% FCS, 25 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.

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.

## ACKNOWLEDGMENTS

We thank the members of Division of Microbiology and Oral Infection for helpful comments and discussions. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to Hotokezaka (#15592169) and Matsuo (#DC2-16-6032).

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J Periodont Res 2007; 42: 541–545
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JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2007.00979.x

# Evidence for association between a Toll-like receptor 4 gene polymorphism and moderate/severe periodontitis in the Japanese population

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*Fukusaki T, Ohara N, Hara Y, Yoshimura A, Yoshiura K. Evidence for association between a Toll-like receptor 4 gene polymorphism and moderate/severe periodontitis in the Japanese population. J Periodont Res 2007; 42: 541–545. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard*

*Background and Objective:* Chronic periodontitis is an inflammatory disease caused by bacteria in subgingival pockets. Because Toll-like receptor 2 and Toll-like receptor 4 have been shown to play an important role in the recognition of periodontal pathogens, we investigated the relevance of genetic variations in *TLR2* and *TLR4* to susceptibility to periodontitis.

*Material and Methods:* A total of 97 patients with chronic periodontitis and 100 control subjects were examined for mutations in *TLR2* and *TLR4*. Case-control analysis was performed using individual single nucleotide polymorphisms detected during the mutation search.

*Results:* The missense mutations reported previously in *TLR2* (677 Arg > Trp and 753 Arg > Gln) and in *TLR4* (299 Asp > Gly and 399 Thr > Ile) were not detected in 97 of the Japanese patients with chronic periodontitis or in 100 of the Japanese control subjects. Nine single nucleotide polymorphisms were identified in exons of *TLR2* and *TLR4*. The case-control analysis revealed that the frequency of the C/C genotype at base-pair position +3725 in *TLR4* was significantly higher in both the moderate and the severe periodontitis patient group than in the control group.

*Conclusion:* A genetic variation of *TLR4* might be associated with moderate and severe periodontitis in the Japanese population.

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Key words: chronic periodontitis; polymorphism; Toll-like receptor 2; Toll-like receptor 4

Accepted for publication November 23, 2006

Chronic periodontitis is an inflammatory disease caused by bacterial colonization in the subgingival area (1). The bacteria and their cell wall components can trigger activation of the host immune system through pattern-recognition receptors to induce inflammatory mediators, leading to the destruction of periodontal tissue. Toll-like receptor 2 and Toll-like receptor 4 are two principal pattern-recognition receptors dedicated to the recognition of bacterial cell wall components, such as lipoproteins and lipopolysaccharides (2,3). We previously demonstrated that Toll-like receptor 2 and Toll-like receptor 4 are involved in the recognition of periodontopathic bacteria, such as *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* (4), and that the expression of those two receptors is augmented in connective tissue subjacent to the periodontal epithelium in patients with severe periodontitis (5). These findings suggest that Toll-like receptor 2 and Toll-like

receptor 4 may be involved in the pathogenesis of periodontal diseases.

It has been reported that two missense mutations in *TLR4* (D299G and T399I) are associated with endotoxin hyporesponsiveness. The allele frequency of the D299G mutation was demonstrated to be 3–8% in European and American populations, and these mutations were associated with a blunted response of the subjects to inhaled lipopolysaccharide (6). Two missense mutations in *TLR2* (R677W and R753Q) were later identified (7,8). Although the R753Q mutation occurred in ≈ 3% of healthy subjects, the mutation was detected at a higher frequency (9%) in patients with gram-positive septic shock in France and was associated with a significantly reduced response to bacterial lipoprotein. The R677W mutation was detected in 10 of 45 lepromatous leprosy patients in Korea, but not found in 41 tuberculoid leprosy patients or in 45 healthy controls.

Recently, the association between these four mutations and periodontitis has been investigated. Folwaczny *et al.* found no association between chronic periodontitis and the missense mutations in *TLR2* and *TLR4* (9), and Laine *et al.* also demonstrated that the missense mutations in *TLR4* were not associated with severe periodontitis (10). On the other hand, Schröder *et al.* reported a positive association between periodontitis and the missense mutations in *TLR4* (11). They reported that patients suffering from chronic periodontitis showed a higher frequency of the missense mutations (D299G/T399I) than controls, and that the genotypes with D299G or T399I were found exclusively in patients, whereas no difference was observed for *TLR2* (11). Brett *et al.* reported, conversely, that the *TLR4* T399I minor allele was more frequent in controls than in patients with aggressive and chronic periodontitis (12). These complicated results might be explained by differences between populations.

The aim of this study was to determine whether these polymorphisms have any association with periodontitis in the Japanese population. We were unable to detect any of these four missense mutations in *TLR2*/*TLR4* in 197 Japanese subjects. Therefore, we attempted to identify single nucleotide polymorphisms in *TLR2* and *TLR4* in Japanese periodontitis patients, and we performed association analysis, using single nucleotide polymorphisms in *TLR2*/*TLR4*, to periodontitis. We show here the association between one single nucleotide polymorphism, in *TLR4* and severe/moderate periodontitis in Japanese subjects.

*Table 1.* Classification of periodontitis patients and controls

| | | Classification of periodontitis | | |
|---|---|---|---|---|
| | Controls | Mild | Moderate | Severe |
| Bone loss ≥ 50% | – | – | ≤ 3 teeth | ≥ 4 teeth |
| No. of subjects | 100 | 16 | 65 | 16 |
| Maximum PPD (mm) | 2.95 ± 0.22 | 4.13 ± 0.50 ($p < 0.01$) | 6.15 ± 0.71 ($p < 0.001$) | 10.50 ± 1.46 ($p < 0.001$) |

Periodontitis patients were assigned to one of three groups of disease severity on the basis of the above criteria.
$p$-values were calculated in comparison to the control group.
PPD, probing pocket depth.

## Material and methods

### Subjects

Patients with chronic periodontitis (59 women and 38 men) and healthy subjects (53 women and 47 men), who visited Nagasaki University Hospital, were enrolled in this study. All of the subjects were Japanese, resided in or around Nagasaki, and had more than 20 teeth. Individuals with malignant diseases, immunodeficiencies, pregnancy, diabetes mellitus, or who had infectious diseases, such as acquired immunedeficiency syndrome or adult T-cell leukemia, were excluded. The mean age of the patients was 60 years (range: 36–83 years) and that of the control population was 46 years (range: 25–75 years). The subjects were screened by full-mouth radiographic assessment and periodontal examinations. Subjects who had neither alveolar bone loss of > 25%, nor periodontal attachment loss of > 3 mm at any sites, were classified into the control group. Subjects who had alveolar bone loss of > 25%, or periodontal attachment loss of > 3 mm in at least at one site, were classified into the chronic periodontitis group. Periodontitis patients were further classified into three groups on the basis of the criteria of disease severity described in Table 1. To make a comparison with a group in the same age range, periodontitis patients were classified into two groups. Patients who were < 60 years of age were categorized into the younger periodontitis patient group, and patients ≥ 60 years of age were categorized into the older periodontitis patient group. The mean age of the younger periodontitis patients was 49 years and that of the older periodontitis patients was 70 years. There was no significant difference between the ages of the younger periodontitis patients and those of the control subjects. Written informed consent was obtained from all of the participants in this study.

### Detection of missense mutations in *TLR2* (R677W and R753Q) and *TLR4* (D299G and T399I)

DNA was extracted from peripheral blood leukocytes by the phenol–chloroform method and harvested by ethanol precipitation. Genotyping of *TLR2* (R677W and R753Q) and *TLR4* (D299G and T399I) was accomplished with the polymerase chain reaction (PCR) and restriction enzyme digestion, following the procedures described by Schröder *et al.* (13) and Lorenz *et al.* (14), respectively.

### Determination of polymorphisms/mutations in *TLR2 and TLR4*

In order to identify single nucleotide polymorphisms peculiar to the Japanese patient group, we performed direct sequencing of all the exons and introns of *TLR2* and *TLR4* (15) in 16 patients with severe periodontitis. PCR ampli-

fication was performed at various annealing temperatures using Takara ExTaq™. PCR products were sequenced using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit v3.1 (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3100 automated sequencer (Applied Biosystems). Sequencing electropherograms were aligned by ATGC software, version 3.0 (Genetyx, Tokyo, Japan), and base alterations were inspected visually.

### Statistical calculation for the case-control study

As a result of single nucleotide polymorphism/mutation detection, we found five single nucleotide polymorphisms in *TLR2* and four single nucleotide polymorphisms in *TLR4*. Next, we performed genotyping of all of those single nucleotide polymorphisms in the remaining 81 patients and 100 controls. Individual single nucleotide polymorphisms were tested for Hardy–Weinberg distribution and linkage disequilibrium using SNPALYZE software (Dynacom, Yokohama, Japan). Case-control analysis was performed at individual single nucleotide polymorphisms using SNPALYZE software. The values of D′ and $r^2$ were calculated and referred for haplotype analysis.

## Results

We failed to identify any of the reported mutations – R677W and R753Q in *TLR2* and D299G and T399I in *TLR4* – in 197 Japanese subjects, comprising 97 patients with periodontitis and 100 healthy controls.

To examine an association between *TLR2*/*TLR4* and periodontitis, we needed to find single nucleotide polymorphisms in *TLR2* and *TLR4*. Therefore, we decided to perform direct sequencing of *TLR2* and *TLR4* in 16 of the patients with severe periodontitis in the present study. Three point mutations in the 5′-untranslated region, and two synonymous mutations in the coding region of *TLR2*, were identified at base-pair positions −183, −148, −146, +1350 (corresponding to rs3804100) and +2343 (corresponding to rs5743709). Four point mutations were identified in the 3′-untranslated region of *TLR4* at base-pair positions +3528, +3725 (corresponding to rs11536889), +4022 and +4529 bp (Table 2). None of the mutations resulted in amino acid substitution. Seven of the nine single nucleotide polymorphisms were present at a frequency of <2% in patients and therefore would provide only a low power of association, but we performed association analysis using all of the nine single nucleotide polymorphisms. The case-control analysis revealed a significant difference between the genotype frequency of the mutation at base-pair position +3725 in *TLR4* of the patient group with that of the control group ($p = 0.043$) (Table 3). There was no significant difference between the frequencies of the other eight single nucleotide polymorphisms. Next, the genotype frequency at base-pair position +3725 in *TLR4* of the control group was compared with the genotype frequencies in the groups of patients with mild, moderate and severe periodontitis. There was no significant difference between the control group and the mild periodontitis patient group, but significant differences were found between the control group and the moderate/severe periodontitis patient groups, as

*Table 2.* Minor allele frequencies of single nucleotide polymorphisms in TLR2 and TLR4 genes

| Gene and SNP position | SNP ID in NCBI | Nucleotide change in major/minor alleles | Minor allele frequency | | HWE |
|---|---|---|---|---|---|
| | | | Controls | Patients | |
| *TLR2* | | | | | |
| −183 | [a] | A/G | 0 | 0.0053 | |
| −148 | [a] | C/T | 0 | 0.0053 | |
| −146 | [a] | T/G | 0 | 0.0053 | $p > 0.05$ |
| +1350 | rs3804100 | T/C | 0.2447 | 0.1684 | |
| +2343 | rs5743709 | G/A | 0 | 0.0156 | |
| *TLR4* | | | | | |
| +3528 | [a] | C/G | 0 | 0.0105 | $p > 0.05$ |
| +3725 | rs11536889 | G/C | 0.1414 | 0.1959 | |
| +4022 | [a] | C/G | 0 | 0.0053 | |
| +4529 | [a] | G/C | 0.0104 | 0.0053 | |

[a]Novel single nucleotide polymorphism (SNP).
HWE, *p*-value of the Hardy–Weinberg equilibrium test in the control population. (The Hardy–Weinberg equilibrium test was performed for only two single nucleotide polymorphisms because minor allele frequencies of other single nucleotide polymorphisms were < 0.05.); ID, identity; NCBI, National Center for Biotechnology Information.

*Table 3.* Case-control analysis using single nucleotide polymorphisms in TLR2 and TLR4 genes

| Gene and SNP position | Genotype frequency (%) | | | | | | *p*-value |
|---|---|---|---|---|---|---|---|
| | Controls | | | Patients | | | |
| | MM | Mm | mm | MM | Mm | mm | |
| *TLR2* | | | | | | | |
| −183 | 93 (100) | 0 (0) | 0 (0) | 90 (99) | 1 (1) | 0 (0) | 0.311 |
| −148 | 93 (100) | 0 (0) | 0 (0) | 90 (99) | 1 (1) | 0 (0) | 0.311 |
| −146 | 93 (100) | 0 (0) | 0 (0) | 90 (99) | 1 (1) | 0 (0) | 0.311 |
| +1350 | 56 (60) | 30 (32) | 8 (8) | 66 (72) | 21 (23) | 5 (5) | 0.214 |
| +2343 | 100 (100) | 0 (0) | 0 (0) | 93 (97) | 3 (3) | 0 (0) | 0.082 |
| *TLR4* | | | | | | | |
| +3528 | 97 (100) | 0 (0) | 0 (0) | 93 (98) | 2 (2) | 0 (0) | 0.151 |
| +3725 | 73 (74) | 24 (24) | 2 (2) | 69 (71) | 18 (19) | 10 (10) | 0.043 |
| +4022 | 92 (100) | 0 (0) | 0 (0) | 92 (99) | 1 (1) | 0 (0) | 0.319 |
| +4529 | 94 (98) | 2 (2) | 0 (0) | 93 (99) | 1 (1) | 0 (0) | 0.573 |

SNP, single nucleotide polymorphism.

a recessive effect ($p = 0.016$ for the moderate periodontitis patient group and $p = 0.034$ for the severe periodontitis patient group) (Table 4). No significant difference was found between the control group and the mild/moderate/severe periodontitis patient groups regarding the frequencies of the other eight single nucleotide polymorphisms in *TLR2* and *TLR4*.

Because the mean age of the control group was significantly younger than that of the patient group, we classified the patients into two groups, according to their ages, to enable comparison with the group in the same age range. The frequency of the 'C/C' genotype at the +3725 base-pair position in *TLR4* in the group of younger periodontitis patients was significantly higher than that in the control group ($p = 0.022$), whereas no significant difference was found between subjects in the older periodontitis patient group and the control group (Table 5).

## Discussion

The missense mutations, reported previously, in *TLR2* (R677W and R753Q) and *TLR4* (D299G and T399I), were not found in the present study (6–8). These results are consistent with the report that the D299G mutation in *TLR4* is not present in the Japanese (16) and Chinese populations (17). Because three of the four missense mutations (R753Q in *TLR2*, and D299G and T399I in *TLR4*) were reported only in European and American populations, the reason why our present results are inconsistent with previous reports might come from the differences between populations. Although R677W in *TLR2* was identified in Korean lepromatous leprosy patients, it was not detected in 286 Indian lepromatous leprosy patients (18). It was suggested that the R677W mutation might come from the variation in the duplicated region with 93% homology to *TLR2* exon 3 located at ≈23 kb 5′-position to the functional *TLR2* gene (18). Because the primers we used in this study were designed specifically for the functional *TLR2* gene, our results are definitive. Although there are conflicting results in the literature regarding the association between those four missense mutations and the susceptibility to periodontitis (9–12), it is difficult to use those four missense mutations to confirm the association between periodontitis and *TLR2*/*TLR4* in the Japanese population because those missense mutations are very rare in Japanese subjects.

We searched for single nucleotide polymorphisms around exons in *TLR2*/*TLR4* because it is possible that other single nucleotide polymorphisms, previously reported, are associated with periodontitis in Japanese subjects. We found nine single nucleotide polymorphisms in the exons of *TLR2* and *TLR4* in the present study; however, none resulted in amino acid substitution. A missense mutation(s) in *TLR2*/*TLR4* would probably not be found as a common variation in Japanese periodontitis patients. It is possible that periodontitis is based on many rare variants, although we did not perform a mutation search in *TLR2*/*TLR4* in all of the patients. Smirnova *et al.* reported that 11 rare missense mutations in *TLR4* were found in 197 meningococcal patients, but that only one rare missense mutation was identified in 127 controls in the UK (19). This is an example that is consistent with the hypothesis that many rare variants are related to common diseases.

We found that the *TLR4* +3725G>C mutation was associated with the whole periodontitis group, and a significant association was also found between the control group and the moderate/severe periodontitis patient groups. Although the mean age of the control group was lower than that of the patient group, the 'C/C' genotype was observed more frequently in the younger periodontitis patient group than in the control group. The positive results from the whole case-control study, and the comparison between the age-matched groups, strongly suggest that the *TLR4* +3725G>C mutation is associated with periodontitis. We did not perform a haplotype association study because the single nucleotide polymorphisms used in this study showed no evidence of linkage disequilibrium (data not shown) with each other. In our next research step, we need to perform a mutation search for other base changes within the genomic region, including *TLR4*. Such a study will uncover the single nucleotide polymorphisms in

*Table 4.* TLR4 +3725 (rs11536889) genotype frequencies in periodontitis patients (mild, moderate, severe) and control subjects

| SNP ID | Subjects | Genotype GG (%) | GC (%) | CC (%) | *p*-value |
|---|---|---|---|---|---|
| rs11536889 | Mild | 9 (63) | 6 (32) | 1 (5) | *P*1 = 0.325<br>*P*2 = 0.151 |
| | Moderate | 47 (71) | 11 (18) | 7 (11) | *P*1 = 0.016<br>*P*2 = 0.840 |
| | Severe | 13 (81) | 1 (6) | 2 (13) | *P*1 = 0.034<br>*P*2 = 0.521 |
| | Control | 73 (74) | 24 (24) | 2 (2) | |

ID, identity; SNP, single nucleotide polymorphism.
*P*1: *p*-value considered as the C allele having a recessive effect (GG + GC vs. CC).
*P*2: *p*-value considered as the C allele having a dominant effect (GG vs. GC + CC).

*Table 5.* TLR4 +3725 (rs11536889) genotype frequencies in the younger periodontitis patient group (< 60 years of age) and in the older periodontitis patient group (≥ 60 years of age)

| Age-group | Genotype GG (%) | GC (%) | CC (%) | *p*-value |
|---|---|---|---|---|
| Younger | 32 (70) | 8 (17) | 6 (13) | 0.022 |
| Older | 37 (72) | 10 (20) | 4 (8) | 0.204 |

*p*-values were calculated in comparison to the control group.

linkage disequilibrium with +3725G >C of the *TLR4* gene or the disease-associated haplotype within the *TLR4* gene. Considering that the progression of periodontitis is affected by multiple factors, such as oral hygiene and the deposition of calculus, a genetic influence may not be sufficient to distinguish the mild periodontitis group from the control group. Age is also known to be a putative risk factor for periodontitis (20), and older patients, with a relatively low-genetic background of mutations, might be suffering from periodontitis. Those factors may account for the lack of statistical difference between the control group and the mild periodontitis group, and between the control group and the older periodontitis group.

Because the +3725G>C mutation is located in the 3′-untranslated region of *TLR4*, it does not have any direct influence on the conformation of the Toll-like receptor 4 protein molecule, according to our present biological knowledge. However, because single nucleotide polymorphisms in introns and/or untranslated regions may influence transcription and/or translation (21–24), the +3725G>C mutation might have a direct effect on mRNA stability or translation efficiency. Antisense transcripts might be important for regulating *TLR4* transcription. The reported disease-associated single nucleotide polymorphisms or haplotypes are not always found in coding regions in 'common diseases' (25,26). The functional assay of disease association with single nucleotide polymorphisms in introns is the next point requiring investigation. In view of the importance of the Toll-like receptor 4 in the pathogenesis of periodontal diseases, the biological significance of genetic variation, including transcription efficiency of the mutated gene, needs to be elucidated.

## Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, 16390614 (to A.Y.).

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# 結　核

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保健の科学　第49巻　第10号（2007.10）別刷

特集　新興・再興感染症の現状と予防

# 結　核

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## はじめに

結核は代表的な再興感染症であり，世界および日本においては単一病原体感染症として人類に甚大な健康被害を及ぼしている．2004年の世界の感染症による年間死亡者は，総死亡者数約5,700万人の1/4強にあたる約1,490万人である．感染症死亡者数の内訳は表1のとおりである[1]．

世界保健機関（WHO）やG8サミットは，①HIV（ヒト免疫不全ウイルス）／AIDS（後天性免疫不全症候群），②結核，③マラリア（熱帯熱マラリア）による死亡者数が年間約500万人，患者発生が約3億人であることから，これら3大疾患を最重要感染症に認定し，世界が協調して対策を構築することを宣言している．

表1　世界における感染症による死亡者数(2004)
(Morens, 2004,[1] より引用改変)

| 感染症 | 死亡数 |
|---|---|
| 急性呼吸器感染症 | 396 |
| AIDS（結核合併を含む） | 277 |
| 下痢性疾患 | 180 |
| 結核 | 156 |
| マラリア | 127 |
| ワクチン予防可能小児疾患 | 112 |
| その他 | 242 |
| 全感染症 | 1,490 |

単位：万人

## 1．結核の発生動向

2007年現在，世界では，全人口の約1/3にあたる約20億人が結核菌（*Mycobacterium tuberculosis*）に既感染（ほとんどは潜在性）し，毎年約880万人が結核を発病，約200万人（AIDS合併を含む）が死亡し，有病者は約2,200万人である[2]．すなわち，数秒ごとに新規結核患者が発生し，15秒ごとに1人が結核で死亡，1人の無治療結核患者が年間10～15人の感染者を生じさせ，毎年世界の人口の約1%が結核菌に感染している．なお，結核菌感染後の生涯にわたる発病率は5～10%である．WHOは，今後20年間で約10億人の新規感染者が発生，約1億5,000万人が結核を発病，そして，約3,600万人の結核死亡者を予測している．

2005年，日本では年間約2万8,000人（罹患率：人口10万対22.2人）が結核を発病，約2,300人（死亡率：人口10万対1.8人）が死亡し，結核は甚大な感染症である（表2）[3]．

日本における結核対策の課題として，①急速な

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表2 世界および日本の結核発生動向(厚生労働省, 2005, [3])

| | 結核菌既感染者数 | 年間死亡者数 | 新規登録患者数 | 有病者数 |
|---|---|---|---|---|
| 世界 | 20億人 | 200万人 | 880万人 | 2,200万人 |
| 日本 | 0.3億人 | 0.23万人 | 2.8万人 | 2.4万人 |

人口の高齢化に伴う高齢者結核の増加(70歳以上の占める割合は約45%), ②国内地域格差の拡大(最高罹患率は大阪市で58.8, 最低は長野県で10.7), ③集団や院内感染の続発および増加(1999〜2004年の合計は220件, 結核集団感染の定義は, 同一の感染源が2家族以上にまたがり, 20人以上に結核を感染させた場合. なお, 発病者1人は感染者6人と換算する), ④多剤耐性結核菌の出現(初回治療耐性: 1〜2%, 再治療耐性: 10〜20%, 多剤耐性結核菌の定義は, イソニアジド(INH)とリファンピシン(RIF)に少なくとも同時耐性), ⑤特異的, 迅速かつ簡便な結核菌感染の検査法の開発, ⑥潜在性結核菌感染対策などがある. 加えて, HIV/AIDSが着実に増加している現状を考慮した場合, 日本においてもHIV/AIDSと結核の重複感染は将来的に重要な課題となることが想定される.

また, 世界と共通した重要な課題としては, ①多剤耐性結核(MDR-TB)の出現, ②HIV/AIDS, ③潜在性結核菌感染対策である[4]. 最近, 超多剤耐性結核菌(XDR-TB, MDR-TBに加え, フルオロキノン耐性+カナマイシン, カプレオマイシン, アミカシンの1剤以上に耐性)も出現している.

薬剤耐性結核の出現を防止する効果的な戦略は薬剤感受性結核を確実に治療し, 治癒させることであり, WHOは直接監視下短期抗結核化学療法(Directly Observed Treatment, Short course: DOTS)を推奨している. DOTSの基本は標準的な抗結核化学療法薬として, イソニアジド, リファンピシン, エサンブトール(EMB)およびピラジナミド(PZA)の4薬を併用し, かつ, 患者の服薬を毎日確認することである(面前での服用も含む).

表3 内因性再燃における発病相対危険度

| 状況 | 発病相対危険度 |
|---|---|
| HIV感染者 | 9.4〜9.9 |
| 陳旧性結核 | 5.2 |
| 慢性腎不全 | 2.4 |
| 抗サイトカイン療法(IL-1拮抗薬, TNF抗体や可溶性受容体) | 2.0 |
| 糖尿病(管理不良) | 1.7 |
| 珪肺症 | 1.2〜1.7 |
| 低体重(基準値の10%以上のるいそう) | 1.6 |
| 胃切除 | 1.4 |

AIDSを含む世界のHIV感染者数は約4,000万人, 結核菌とHIVの重複感染者数は約1,500万人, 結核を発症した患者でHIV陽性は約8%を占めている[5]. 結核菌感染に対する宿主防御は細胞性免疫(マクロファージ—サイトカイン—1型ヘルパーTリンパ球連関)の発現に依存しているが, HIV/AIDSは細胞性免疫を破壊するため, 結核菌感染や発病を惹起しやすくする. 実際, HIV陽性者の発病の相対危険度はHIV陰性者の約10倍である. また, AIDS死亡の約10%が結核を直接原因としている.

結核の発病様式は, 前述した全人口の約1/3にあたる約20億人が感染している①潜在性結核菌感染を起源とした内因性再燃と②外来性再感染に大別される. 結核菌感染後の発病率は5〜10%であるが, その多くは①内因性再燃によるものである. したがって, 潜在性結核菌感染者を科学的・効率的に発見し, 表3に示した発病高危険群や濃厚接触者などの潜在性感染者に治療(発病予防)介入することは, 結核制圧に新戦略を提供するであろう[6].

## 2. 結核菌の生物学的特徴や病原性

表4[7]に示したように, 結核菌の生物学的特徴として, ①細胞内寄生性, ②脂質成分に富む細胞壁, ③好気性, ④遅発育性, ⑤空気(飛沫核)感染, ⑥慢性炎症, ⑦遺伝子の解読などがある. 結核菌は好気性グラム陽性桿菌, 細胞内寄生病原体