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Induction of *Porphyromonas gingivalis* GroEL signaling via binding to Toll-like receptors 2 and 4

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Background/aims: Heat shock protein 60 (HSP60) has been recognized as an important molecule in infectious and autoimmune diseases. Although *Porphyromonas gingivalis* GroEL, a homologue of HSP60, is a potent stimulator of inflammatory cytokines, its receptor and signaling mechanisms are not yet understood in detail. In this study, we investigated whether the Toll-like receptor (TLR) family plays a functional role as a *P. gingivalis* GroEL receptor.
Methods: Human macrophage-like THP-1 cells were used and the nuclear factor-κB (NF-κB) activity of cells stimulated with a recombinant *P. gingivalis* GroEL was measured with a luciferase assay. Flow cytometry analysis was used to determine the binding to THP-1 cells of fluorescein isothiocyanate (FITC)-labeled GroEL. In addition, anti-human TLR (anti-hTLR)2 and anti-hTLR4 monoclonal antibodies were used to assess the functional role of TLR2 and TLR4 as the receptors for GroEL.
Results: We observed by luciferase assay that the purified recombinant GroEL was able to stimulate NF-κB transcriptional activity in THP-1 cells. Flow cytometry analysis showed that the FITC-labeled GroEL bound to THP-1 cells in a dose-dependent fashion. Our binding competition analysis with FITC-labeled and unlabeled GroEL showed that it bound to the cells as a specific mode of action. On the other hand, GroEL-stimulated NF-κB transcriptional activity was significantly inhibited by anti-hTLR2 and anti-hTLR4 antibodies and was inhibited more strongly by a combination of both antibodies.
Conclusion: Our present study demonstrates that *P. gingivalis* GroEL induces its intracellular signaling cascade in THP-1 cells via TLR2 or TLR4 and via a combination of both receptors.

Key words: GroEL; nuclear factor-κB; *Porphyromonas gingivalis*; Toll-like receptor

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Heat shock proteins (HSPs), which are highly conserved through evolution, are molecular chaperons that bind non-native states of other proteins and assist them in obtaining their functional conformation (7, 10, 14). In addition, they have an important function in folding newly synthesized proteins and preventing their aggregation and misfolding. Among HSP families, GroEL, a homologue of the HSP60 family, is a major HSP in various bacterial infections (19), and is widely recognized as an important molecule in infectious and autoimmune diseases (32).

Porphyromonas gingivalis, a gram-negative anaerobe, is frequently found in the subgingival flora of periodontitis patients and contributes to periodontal disease pathogenesis (23). Many studies (1, 15, 21, 24, 27) have demonstrated that *P. gingivalis* cell surface components, such as lipopolysaccharide (LPS) and fimbriae, are important pathogenic components involved in the initiation and development of periodontal diseases. These components are potent stimulators of inflammatory cytokine production and bone resorption (2, 5, 15, 17, 21, 24). Several studies (11, 26, 28, 31, 37) have reported that some bacterial HSPs stimulate the production of

pro-inflammatory cytokines by human monocytes and also upregulate the expression of adhesion molecules (12, 34); GroEL of *P. gingivalis* and *Actinobacillus actinomycetemcomitans* are also able to stimulate the expression of pro-inflammatory cytokines in macrophages (17). However, the intracellular signaling mechanism utilized by GroEL has not yet been demonstrated in detail.

Recent studies (4, 8, 15, 16, 24) have demonstrated that Toll-like receptors (TLRs), homologues of the *Drosophila* Toll gene, recognize bacterial cell components such as LPS, peptidoglycans, and lipopeptides, as well as flagella, bacterial DNA, and viral double-stranded RNA. Following this recognition, the signal transduction is initiated by TLRs. Thus, we hypothesized that *P. gingivalis* GroEL induces its intracellular signaling via binding to a member of the TLR family.

In this study, we show that *P. gingivalis* GroEL binds to human macrophage-like THP-1 cells via TLR2 and TLR4 and that its intracellular signaling is induced by stimulation of both receptors.

Materials and methods

Reagents

Lipopolysaccharide, fluorescein isothiocyanate (FITC)–LPS from *Escherichia coli* O111:B4 and lipoteichoic acid (LTA) from *Bacillus subtilis* were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO). The *Limulus* ES-II single test was supplied by the Wako Pharmaceutical Co. (Osaka, Japan). Affi-Prep polymyxin matrix was obtained from Bio-Rad Laboratories (Richmond, CA). Anti-human TLR2 (anti-hTLR2) mouse monoclonal antibody was purchased from Cascade Bioscience (Winchester, MA) and anti-human TLR4 (anti-hTLR4) mouse monoclonal antibody was from Serotec (Oxford, UK). Functional grade purified mouse immunoglobulin G2a (IgG2a) isotype control monoclonal antibody was supplied by eBioscience (San Diego, CA). Xpress System protein purification and ProBond resin were obtained from Invitrogen Corp. (Carlsbad, CA) and the Polyfect transfection reagent was from Qiagen K. K. (Tokyo, Japan). The dual-luciferase reporter assay system was supplied by Promega (Madison, WI) and the FITC label kit was from American Qualex (San Clemente, CA).

Expression plasmids

The expression plasmid for *P. gingivalis* GroEL (pTEL) was constructed in the following manner. Briefly, the plasmid encoding *P. gingivalis* GroEL (pMEL), provided by Dr Hotokezaka (Nagasaki University, Nagasaki, Japan), was digested with *Sac*I and *Hin*dIII restriction enzymes. The DNA fragment encoding GroEL was cloned into the *Sac*I and *Hin*dIII sites of the vector plasmid pTrcHisB (Invitrogen), which generated plasmid pTEL. Luciferase reporter plasmid (pTKκB2luc) and luciferase internal control plasmid (pRL-TK), containing *Renilla* luciferase cDNA, were provided by Dr Ohmori (Meikai University, Saitama, Japan). All plasmids were purified using the EndoFree plasmid kit (Qiagen) according to the manufacturer's recommendations.

Purification of recombinant *P. gingivalis* GroEL

P. gingivalis GroEL was purified using Xpress System protein purification (Invitrogen) according to the manufacturer's recommendations. Briefly, *E. coli* DH5α containing the pTEL plasmid was inoculated into Luria–Bertani medium supplemented with 50 μg/ml ampicillin (Luria-Bertani medium supplemented with Ampicillin) and incubated overnight at 37°C. The overnight culture (5 ml) was inoculated into 500 ml fresh LBA medium and grown until an optical density of 0.5 at 540 nm was obtained. The cells were treated with isopropyl β-D-thiogalactopyranoside (Sigma-Aldrich) at a final concentration of 1 mmol and then incubated for another 2 h with vigorous shaking. Next, the cells were harvested, resuspended in native binding buffer (20 mmol sodium phosphate, 500 mmol sodium chloride, pH 7.8), and sonicated. These sonicated-cell extracts were subjected to centrifugation and the supernatant was loaded on a ProBond resin column. This column was washed five times with native wash buffer (20 mmol sodium phosphate, 500 mmol sodium chloride) at pH 6.0 and 5.5, respectively, and the GroEL protein was eluted with native-pH elution buffer (20 mmol sodium phosphate, 500 mmol sodium chloride, pH 4.0). Then, GroEL was cleaved from the His-tag with factor Xa protease (Promega) and was purified using ProBond resin. Finally, the purified GroEL was passed through Affi-Prep polymyxin matrix for removal of any contaminating endotoxin. The purity of the recombinant GroEL was analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the endotoxin level was measured using the *Limulus* ES-II single test.

FITC labeling of purified *P. gingivalis* GroEL

The *P. gingivalis* GroEL, purified as described above, was FITC-labeled using a FITC labeling kit (American Qualex) according to the manufacturer's recommendations. Briefly, 500 μl FITC (1 mg/ml) was added to 2.5 mg protein suspended in carbonate saline buffer and then mixed by end-over-end rotation for 2 h at room temperature. This solution was passed through a NAP10 column (Amersham Bioscience Corp., Piscataway, NJ) to remove unbound FITC. Finally, the FITC-labeled GroEL (FITC-GroEL) was dialysed overnight against phosphate-buffered saline (pH 7.3).

Cell culture

The human monocytic THP-1 cell line was purchased from the Riken Cell Bank (Tsukuba, Japan) and was grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cell cultures were maintained at 37°C under 5% CO_2.

Luciferase reporter assay

THP-1 cells (3×10^6 cells/plate) were plated on 10-cm dishes and were transiently transfected by using 80 μl PolyFect transfection reagent (Qiagen), pTKκB2luc (8 μg), and pRL-TK (8 μg) per plate. Twenty-four hours after transfection, the cells were harvested, seeded onto a 24-well plate (0.5×10^6 cells/well), and stimulated for 5 h with purified *P. gingivalis* GroEL at different concentrations. After cell lysis, nuclear factor-κB (NF-κB) luciferase activity was measured using the dual-luciferase reporter assay system (Promega). The reporter NF-κB activity was normalized to *Reinilla* luciferase activity. Data are expressed as the fold increases in relative light units (which represent the ratio of pTKκB2luc-luciferase to pRL-TK *Renilla*-luciferase expression) relative to that of the unstimulated control. All experiments were performed in triplicate.

Binding assay for FITC-labeled *P. gingivalis* GroEL to THP-1 cells

THP-1 cells were harvested and washed twice with ice-cold Hanks' balanced salt solution (HBSS; GibcoBRL, Carlsbad, CA). Aliquots containing 1×10^6 cells were placed in flow cytometer test tubes

and 0.5 ml of HBSS supplemented with 1% FBS was then added. Cell suspensions were placed on ice for 30 min and then washed with ice-cold HBSS. FITC-labeled GroEL or FITC-labeled LPS was added to each tube at different concentrations and incubated for another 30 min on ice. Finally, the cells were washed and resuspended in 500 µl of HBSS. The stained cells were analysed using an EPICS XL flow cytometer (Beckman Coulter Inc., Miami, FL).

Binding competition assay of *P. gingivalis* GroEL to THP-1 cells

For the competition assays, THP-1 cells (1×10^6 cells/tube) were preincubated for 30 min on ice in HBSS supplemented with 1% FBS. Then, the cells were washed with cold HBSS and FITC-labeled GroEL (30 µg) or unlabeled GroEL (150 µg) were simultaneously added to the cells and the mixtures were incubated for another 30 min on ice. Subsequently, the cells were washed and resuspended in 500 µl of HBSS. A similar competition assay was performed using FITC-labeled and unlabeled LPS as a control (data not shown). The samples were evaluated using an EPICS XL flow cytometer (Beckman Coulter).

Blocking of TLR2 and TLR4 for *P. gingivalis* GroEL binding to THP-1 cells

THP-1 cells were transfected as described above. Then, these transfected cells (3.5×10^5 cells/well) were seeded in each well of a 24-well plastic plate and incubated for 1 h at 37°C with or without anti-hTLR2 (12 µg) and/or anti-hTLR4 (12 µg) antibodies. The antibody-treated cells were then stimulated for 5 h with or without *P. gingivalis* GroEL. The cells were harvested and then lysed using passive lysis buffer (Promega); their NF-κB transcriptional activities were measured with a dual-luciferase reporter assay system (Promega).

Statistical analysis

Data are presented as the mean ± standard error for at least three independent experiments. Statistical significance was analysed with the Student's *t*-test. Values with $P < 0.05$ were considered significant.

Results

Porphyromonas gingivalis GroEL induces NF-κB transcriptional activity in THP-1 cells

First, to examine whether *P. gingivalis* GroEL is able to stimulate intracellular signaling in human monocytic THP-1 cells, NF-κB transcriptional activity was measured using a NF-κB luciferase assay. As shown in Fig. 1, GroEL strongly stimulated the NF-κB transcriptional activity of the cells in a dose-dependent fashion. These results suggest that GroEL is able to initiate intracellular signaling via binding to its receptor(s) on the THP-1 cells.

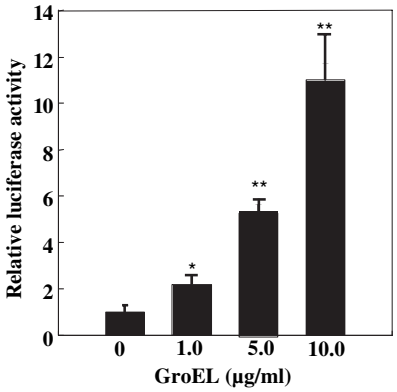

Fig. 1. Porphyromonas gingivalis GroEL induces nuclear factor-κB transcriptional activation in THP-1 cells. Human monocytic THP-1 cells were transfected with pTKκB2luc and pRL-TK, stimulated with purified GroEL (1.0, 5.0 and 10 µg/ml) and harvested; the luciferase activities were then analysed. Values shown are the relative luciferase activity (firefly luciferase/*Renilla* luciferase) compared with the unstimulated control response, which was set at 1. The data are expressed as the mean ± SD from triplicate wells from one experiment and are representative of three independent experiments. Values were significantly different from the value for the untreated cells at each concentration for GroEL as determined with the Student's *t*-test (*$P < 0.001$; **$P < 0.0001$).

Porphyromonas gingivalis GroEL-stimulated NF-κB transcriptional activity in THP-1 cells is not the result of LPS contamination

It was important to demonstrate that LPS contamination was not involved in our recombinant GroEL-stimulated NF-κB transcriptional activity in THP-1 cells. For this reason, our test samples, the *P. gingivalis* GroEL and LPS were all pretreated with polymyxin B (20 U/ml), which is a potent inhibitor of LPS (25), before stimulation. Although LPS-stimulated NF-κB transcriptional activity was dramatically inhibited by polymyxin B, such inhibition was not observed in our GroEL-stimulated cells (Fig. 2). Therefore, these observations suggested that GroEL-stimulated NF-κB transcriptional activity is LPS independent. These data strongly showed that *P. gingivalis* GroEL itself has the ability to act as a ligand to activate the intracellular signaling system.

Binding of *P. gingivalis* GroEL to THP-1 cells

Since the previous experiments had demonstrated that GroEL has a functional

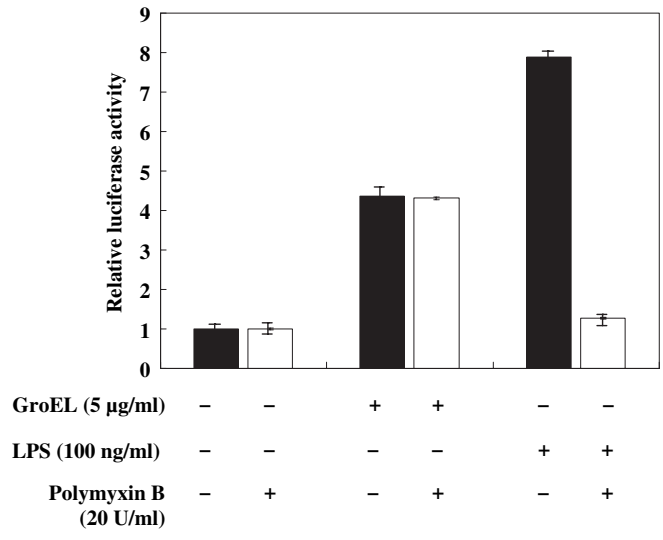

Fig. 2. Porphyromonas gingivalis GroEL-induced signaling in THP-1 cells is not dependent on lipopolysaccharide (LPS) contamination. THP-1 cells were transfected with pTKκB2luc and pRL-TK as indicated. After 24 h, the cells were stimulated with purified GroEL (5 µg/ml) or LPS (100 ng/ml) in the presence or absence of polymyxin B (20 U/ml). Then, the cells were harvested and luciferase activities were analysed. Results are expressed as mean ± SD in triplicate wells from two separate experiments.

role as a ligand, we next wanted to assess if GroEL was able to bind to the cells as a ligand by using a flow cytometry-based assay. Figure 3 clearly shows that GroEL bound to the cells in a dose-dependent manner. Similarly, LPS as a positive control also bound to the cells (data not shown). These results suggested that GroEL binds to the cells by recognizing its cell receptor(s) as a ligand.

Porphyromonas gingivalis GroEL binds to its specific receptor on THP-1 cells

Based on the observation that GroEL binds to cells in a dose-dependent manner, we next wanted to investigate if GroEL binds to the THP-1 cells via specific receptor(s). To demonstrate this point, GroEL-binding specificity to the THP-1 cells was investigated using a competition assay with FITC-labeled and unlabeled GroEL. As shown in Fig. 4A,B, a high binding activity of FITC-labeled GroEL to the cells was clearly inhibited by the simultaneous addition of FITC-unlabeled GroEL, which prevented the FITC-labeled GroEL from binding to its receptor(s) on the THP-1 cells surface, consequently lowering the percentage of cells detected as bound to FITC-labeled GroEL. We also observed that the unlabeled LPS was able to inhibit FITC-labeled LPS binding; this was used as a control (data not shown). Together with the previous data showing dose-

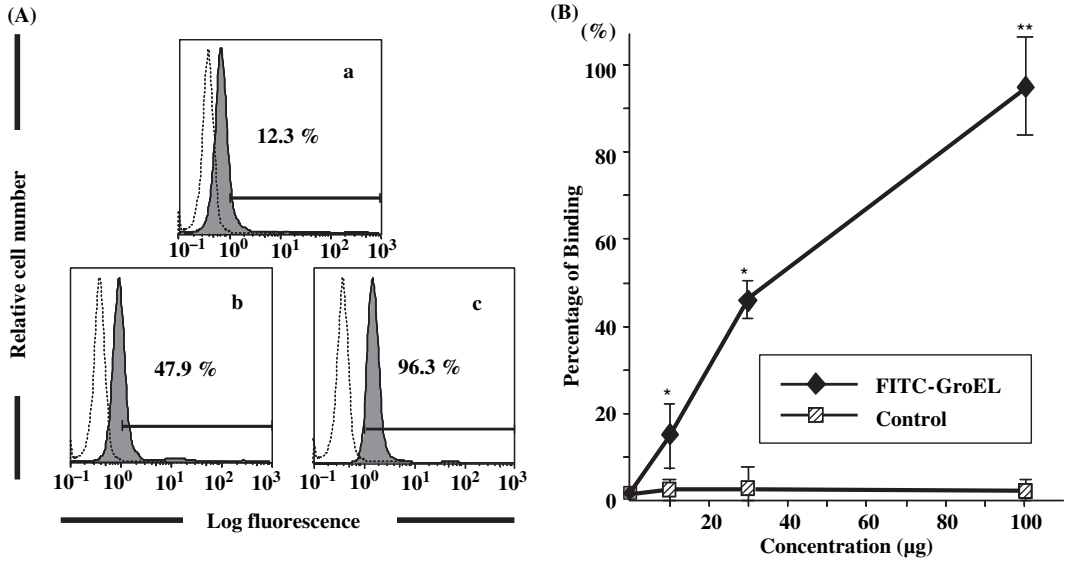

Fig. 3. Direct binding of fluorescein isothiocyanate (FITC)-labeled *Porphyromonas gingivalis* GroEL to THP-1 cells. (A) THP-1 cells were blocked by using Hanks' balanced salt solution supplemented with 1% fetal bovine serum, washed, and then incubated further on ice with FITC-labeled GroEL at 10 µg (a), 30 µg (b) or 100 µg (c) for 30 min. Untreated cells were used as a control (dotted line). Then, the stained cells were analysed by flow cytometry. These results are representative of three independent experiments that had similar results. At least 5000 cells were analysed per experiment. (B) The results are expressed as the mean ± SD from three independent experiments performed in duplicate. Values were significantly different from the value for the untreated cells at each concentration for GroEL as determined with Student's *t*-test (**P* < 0.01; ***P* < 0.0003).

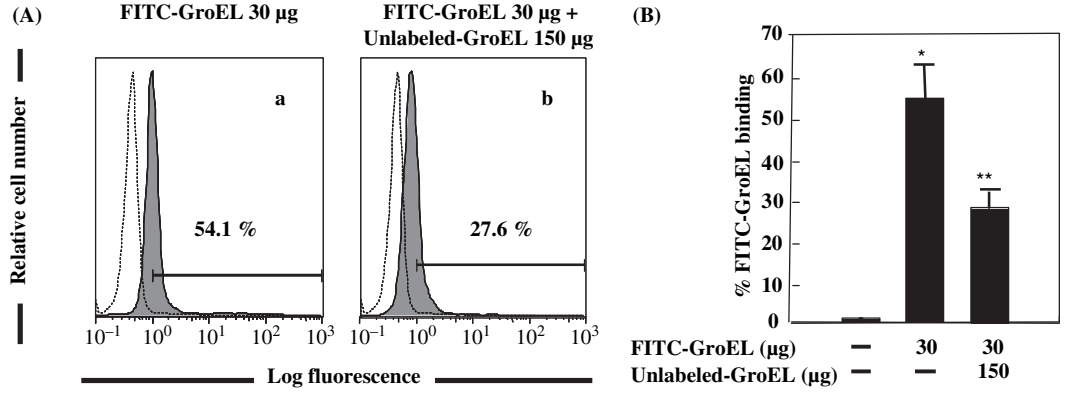

Fig. 4. Fluorescein isothiocyanate (FITC)-labeled *Porphyromonas gingivalis* GroEL binding to THP-1 cells is inhibited by using unlabeled GroEL in a competition assay. (A) THP-1 cells were blocked by using Hanks' balanced salt solution supplemented with 1% fetal bovine serum; washed, and then treated with FITC-labeled GroEL in the absence (a) or presence (b) of unlabeled-GroEL. The cells were incubated on ice and subsequently were analysed using flow cytometry. Untreated cells (dotted lines) were used as a control. (B) The results are expressed as the mean ± SD from two different experiments performed in duplicate. Values using both FITC-labeled and unlabeled GroEL were significantly different from FITC-labeled treated cells (Student's *t*-test; **P* < 0.005, ***P* < 0.001).

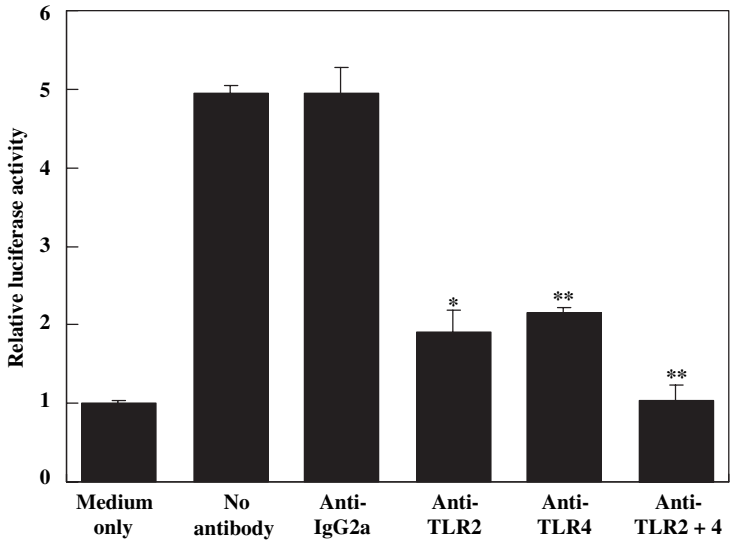

Fig. 5. Porphyromonas gingivalis GroEL-stimulated nuclear factor-κB transcriptional activity in THP-1 cells is inhibited by anti-human Toll-like receptor (anti-hTLR)2 and anti-hTLR4 antibodies. THP-1 cells were transfected as indicated in the Materials and methods. Before addition of the stimulants, the transfected cells were pretreated with anti-hTLR2 antibody, anti-hTLR4 antibody or a combination of both antibodies or were left untreated. Then, the cells were either treated with GroEL or left untreated. Finally, luciferase activities were measured. An anti-mouse IgG2a antibody was used as a negative control. The results are expressed as mean ± SD in triplicate cultures of at least three independent experiments. Student's *t*-test values calculated with respect to the antibody-untreated cells were significantly different (*$P < 0.01$ and **$P < 0.001$).

dependent GroEL binding to THP-1 cells (Fig. 1), these results suggested that *P. gingivalis* GroEL binds to THP-1 cells by recognizing its specific receptor(s) on the THP-1 cell surface.

Anti-hTLR2 and anti-hTLR4 antibodies inhibit *P. gingivalis* GroEL stimulation of NF-κB transcriptional activity in THP-1 cells

Since several studies (4, 9, 25, 30) have shown that the HSP family signals intracellularly via TLR, the NF-κB luciferase assay was used to examine whether GroEL signaling in THP-1 cells also occurs via the TLR family, especially through the family members TLR2 and TLR4. Figure 5 illustrates that the GroEL-stimulated NF-κB transcriptional activity was clearly inhibited by both the anti-hTLR2 and anti-hTLR4 antibodies. This inhibitory action was even greater when a combination of both antibodies was used. To verify the inhibitory effect of both antibodies, the inhibitory effect of each antibody was examined by stimulation of NF-κB transcriptional activity in THP-1 cells treated with LTA and LPS, as positive controls of TLR2- and TLR4-mediated signals, respectively. As expected, the LTA-stimulated NF-κB transcriptional activity was markedly inhibited by the anti-hTLR2 antibody. In addition, the LPS stimulation of the transcriptional activity was inhibited by the anti-hTLR4 antibody (data not shown). For these experiments, an anti-mouse IgG2a monoclonal antibody was used as the control antibody. These results demonstrate that *P. gingivalis* GroEL operates its intracellular signaling effectively using both TLR2 and TLR4, although GroEL is also able to induce its signal through each receptor alone.

Discussion

In the present study, we have demonstrated that *P. gingivalis* GroEL is recognized by both TLR2 and TLR4 and, consequently, its intracellular signaling is induced via both receptors.

Human HSP60 is recognized as a potent activator of murine and human macrophages because this protein induces expression of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-6 and interleukin-12 (6, 18, 36). Several studies (18, 32, 35) have shown that bacterial HSP60 (GroEL) also acts as a powerful stimulator of macrophages. Although a specific receptor(s) that mediates the initiation of GroEL signaling has not been demonstrated in detail, interestingly, many studies (4, 8, 15, 16) have shown that members of the TLR family play an important role in the recognition of bacterial cell components. Based on these data, we investigated whether *P. gingivalis* GroEL is able to activate its intracellular signaling using TLR family members.

The NF-κB/Rel/IκB family of transcription factors regulates a number of genes involved in a wide variety of biological processes. NF-κB is a predominant transcriptional factor that functions in immune and inflammatory responses stimulated by bacterial cell components. We observed that *P. gingivalis* GroEL is a potent stimulator of NF-κB. It has been reported that NF-κB transcriptional activation induced by different HSP60 preparations was the result of LPS and/or LPS-related contaminants in those preparations (13). In the present study, extensive controls excluded this possibility: first, the addition of polymyxin B did not inhibit the *P. gingivalis* GroEL-induced NF-κB; secondly, endotoxin activities in the GroEL preparation were negligible; and thirdly, *P. gingivalis* GroEL was further purified by passage through a polymyxin matrix column.

Many studies (18, 22, 23) have demonstrated that bacterial cell components operate their signaling via binding to their specific receptor. We observed that FITC–GroEL binds to THP-1 cells in a dose-dependent manner, which suggests the presence of a specific receptor(s) for *P. gingivalis* GroEL on the cell surface.

Recent studies (3, 8, 15, 16, 25) have demonstrated that pathogen-associated microbial patterns, including several bacterial cell components, are recognized by the TLR family. TLRs are type I transmembrane proteins with an extracellular domain analogous to that of the interleukin-1 receptor family (20, 29). Together with CD14 and MD-2, TLR4 initiates signaling cascades in response to LPS, whereas TLR2 has been shown to be involved in the recognition of a broad range of microbial products, such as bacterial lipoproteins, LTA and peptidoglycans from gram-positive bacteria (3, 16, 20, 29). Thus, it has been suggested that HSP60 also could be the endogenous ligand for TLR2 and TLR4 (25, 33) and that its protein also serves as a danger signal to the innate immune system through production of endogenous inflammatory cytokines (6). In this study, we have demonstrated that *P. gingivalis* GroEL intracellular signaling in THP-1 cells is clearly inhibited by pretreating the cells with monoclonal antibodies against TLR2 and TLR4, and that such inhibitory action was more effective when the pretreatment used a combination of both antibodies, suggesting an important role

for both TLR2 and TLR4 in the initiation of the GroEL signaling pathway(s) in macrophages. These data are supported by a growing number of studies showing that bacterial components, including *P. gingivalis* LPS (29), are capable of inducing their intracellular signaling system(s) by using both TLR2 and TLR4.

For the binding of the *P. gingivalis* GroEL to TLR2 and TLR4 on macrophages to initiate its intracellular signaling cascade, we speculate the need for a co-stimulatory protein(s) for GroEL to bind to each TLR. This is especially significant because GroEL binding was more effective when the cells were treated in the presence of serum than in the absence of serum (Argueta et al. unpublished data). This observation suggested the presence of a possible co-stimulatory molecule in the serum for GroEL binding and signal transduction. However, because the mode of action for binding and signaling of bacterial cell components via a combination of TLR family members has not been demonstrated in detail, further studies pursuing this specific aspect of the mechanism will be interesting.

In conclusion, our present study demonstrates that *P. gingivalis* GroEL induces its intracellular signaling cascade in THP-1 cells via TLR2 or TLR4 and via a combination of both receptors. As the exact signaling mechanism of GroEL triggered by these TLRs is unknown, further detailed analysis is necessary to reveal the stimulatory mechanism of GroEL signaling.

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