Translocation of *Porphyromonas gingivalis* infected monocytes and sssociated cellular responses

Pongsawat Suwatanapongched[1], Rudee Surarit[1], Ratchapin Srisatjaluk[2], and Steven Offenbacher[3]

Summary

Porphyromonas gingivalis* (*P.gingivalis*), an important periodontal pathogen in adult chronic periodontitis, has been reported to co-localize in human atheromatous lesions. We have studied the phagocytosis and survival of *P.gingivalis* in human monocytes, together with the cellular responses of infected human monocytes. Human monocytes were co-cultured with *P.gingivalis* and the external bacteria were killed with metronidazole and gentamycin. Localization of *P.gingivalis* in cells was studied by transmission electron microscopy (TEM). The survival of *P. gingivalis* was determined by lysing the monocytes and plating on blood agar under anaerobic conditions. Interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) were determined using specific enzyme-linked immunosorbent assays (ELISAs) kits. The transwell chamber system was used to investigate the chemotactic response of the infected cells. TEM showed that *P.gingivalis* organisms were localized within the autophagosome-like structure of monocytes. No significant difference on the survival of *P.gingivalis* at 0, 4 and 8 h after infection was found. IL-1β and TNF-α were present in the cell culture media in response to bacterial challenge. The infected monocytes showed a normal chemotactic response to monocyte chemotactic protein-1 (MCP-1). The number of monocyte cells migrating through membrane in the presence and absence of *P.gingivalis* were $18.64\pm2.33\times10^4$ cells and $19.11\pm1.76\times10^4$ cells respectively. The number of viable *P.gingivalis* per monocyte following translocation in response to the chemotactic gradient was $5.83\pm1.45\times10^{-3}$ CFU/cell. The results indicate that *P.gingivalis* can stimulate cytokine production and survive in monocytes without affecting cell migration. *(Asian Pac J Allergy Immunol 2010;28:192-9)

Key words: *monocyte;* Porphyromonas gingivalis*; TEM; IL-1β; TNF-α; ELISA; monocyte migration; MCP-1*

From the [1]Department of Physiology and Biochemistry, Faculty of Dentistry, Mahidol University, Bangkok, Thailand, [2]Department of Microbiology, Faculty of Dentistry, Mahidol University, Bangkok, Thailand, [3]Dental Research Center, University of North Carolina, Chapel Hill, NC, USA
Corresponding author: Rudee Surarit, Department of Physiology and Biochemistry, Faculty of Dentistry, Mahidol University, Bangkok, Thailand, 10400
E-mail: dtrsr@mahidol.ac.th

Abbreviations:

ATCC = American Type Culture Collection
BHI = agar brain heart infusion agar
CFU = colony forming unit
ddH_2O = double distilled water
***E. coli* =** *Escherichia coli*
ELISAs = enzyme-linked immunosorbent assays
FBS = fetal bovine serum
IL-1β = interleukin-1 beta
JEOL = Japan electron optics laboratory
LPS = lipopolysaccharides
MCP-1= monocyte chemotactic protein-1
MOI = multiplicity of infection
***P.gingivalis* =** *Porphyromonas gingivalis*
PB = phosphate buffer
PBS = phosphate buffer saline
PI 3-kinase = phosphatidylinosotol-3 kinase
RPMI = Roswell Park Memorial Institute medium
RT = room temperature
RT-PCR = reverse transcription-polymerase chain reaction
SD = standard deviation
TEM = transmission electron microscopy
TNF–α = tumor necrosis factor-alpha

Introduction

Porphyromonas gingivalis (P.gingivalis), a gram-negative anaerobic bacterium, is an important periodontal pathogen in adult chronic periodontitis.[1] *In vitro, P.gingivalis* has been reported to invade and survive in human oral cells including buccal epithelium cells[2], oral epithelium cells[3] and gingival epithelium cells[4], as well as in human coronary artery cells.[5] Intracellular invasion may be an important mechanism which allows them to evade the host immune system, phagocytic clearance in particular. *P.gingivalis* infection specifically has been reported to be associated with increased atheroma formation and cardiovascular events.[6] In addition, *P.gingivalis* can promote progression of atherosclerosis in a mouse model[7] and coronary vessel disease in the pig.[8] It is not known how *P.gingivalis* translocates from the periodontal pocket to the subendothelial lining of the major elastic arteries. This tropism may be facilitated by bacteremic seeding, followed by direct adherence to and invasion into the intimal lining of the artery or alternatively, the bacteria may invade cells such as neutrophils or monocytes, which then actively transport the organism to the subendothelial space in a "piggy-back" fashion.

Monocytes play an important role in the human immune response against pathogens, such as phagocytosis, secretion of biologically active molecules and antigen presentation. Monocytes release many mediators including proinflammatory cytokines, e.g. Interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α). These cytokines have various biological activities for example, induction of prostaglandin E_2 secretion, stimulation of neutrophils, inhibition of bone formation, activation of the bone resorption process[9] and promotion of atherosclerosis.[10] In addition, circulating blood monocytes play an important role in the early formation of atherosclerosis by adhering to the vascular endothelium, becoming the dominant cellular infiltrate in the subendothelial compartment. When activated within the subendothelial space, monocytes differentiate into lipid-laden foam cells at the atherosclerotic lesion.[11] *P.gingivalis* also induces the differentiation of monocytes into a foam cell phenotype by increasing the cell receptor density for oxidized low density lipoprotein particles.[12] Many studies have shown that oral bacteria can enter the peripheral blood circulation of periodontal patients through flossing[13], brushing[14] or even eating.[15] Therefore, we hypothesize that *P.gingivalis* can survive in the circulation by residing within monocytes which may carry the bacteria to the site of the atherosclerotic lesion, where they stimulate production of proinflammatory cytokines to facilitate monocytic adherence and subendothelial migration. The objectives of this study were to investigate the ability of *P.gingivalis* to survive in monocytes and to study the chemotactic and translocation responses of phagocytic monocytes.

Methods

Bacterial culture

P. gingivalis strain A7436 was isolated from a patient with refractory periodontitis (by S. Offenbacher) and characterized by the Center for Disease Control and Prevention, Department of Oral Biology, University of Buffalo, Buffalo, NY, USA. The bacteria were grown on anaerobic blood agar (Oxoid, Basingstoke, Hamphire, UK) in an anaerobic chamber (Mitsubishi Gas Chemical, Chiyoda-yu, Tokyu, Japan) with an atmosphere of 10% CO_2, 5% H_2 and 85% N_2 at 37°C for 7 days. *Escherichia coli* (*E.coli*) ATCC 25922 (American Type Culture Collection, Rockville, MD, USA) was grown on Brain Heart Infusion agar (BHI agar) (Becton Dickinson, Sparks, MD, USA), incubated aerobically at 37°C for 24 h. Prior to the experiment, the bacteria were suspended in RPMI (Roswell Park Memorial Institute medium) 1640 medium (Gibco, Grand Island, NY, USA) and the cell density was adjusted to the optical density of 0.1 at 660 nm (1 x 10^8 Colony forming unit/ml or CFU/ml) using a spectrophotometer (Milton Roy, Ivyland, PA, USA).

Cell culture

The human monocytic cell line designated as THP-1 was purchased from ATCC and was maintained in RPMI 1640 containing 10% heat inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2. The cell density of $1x10^6$ cells/ml was used in all experiments.

Bacterial localization in THP-1 cell

P. gingivalis A7436 cells were inoculated into the THP-1 cell suspension at a multiplicity of infection (MOI; the number of bacteria per a host cell) of 100 and incubated at 37°C in 5% CO_2 for 90 min, followed by centrifugation at 137×g for 10 min at 4°C. The cell pellets were then treated

with 300 μg/ml gentamicin and 200 μg/ml metronidazole, centrifuged, and incubated in RPMI 1640 containing 10% heat inactivated FBS at 37°C in 5% CO_2 for 0, 4 and 8 h. THP-1 cells were then prepared for transmission electron microscopy (TEM) (The Japan electron optics laboratory (JEOL), Akishima, Tokyo, Japan). Briefly, cells were fixed in phosphate buffer (PB) containing 4% glutaraldehyde at pH 7.4 for 30 min at room temperature (RT), washed 3 times with PB, followed by fixing in 2% osmium teroxide for 45 min, washing and staining with 2% uranyl acetate for 30 min at RT. Specimens were dehydrated in a graded series of ethanol solutions (70%-100%), followed by propylene oxide twice, for 20 min each. The cells were infiltrated with a mixture of propylene oxide and epoxy resin at the ratio of 1:1 at 37°C for 30 min and with the resin at 37°C for 2 h. After being polymerized at 70° C overnight, sections of 60-90 nm were cut using an ultramicrotome. The thin sections were collected and mounted on copper grids, stained with 5% uranyl acetate in 70% methanol, followed by lead citrate for 30 min each, and then examined by TEM. For studying extracellular bacterial attachment, specimens were made from the infected THP-1 cells without antibiotics treatment. Non-infected THP-1 cells treated with antibiotics were included as a control in all experiments.

Survival of P.gingivalis in THP-1 cells

The viability of intracellular *P.gingivalis* at MOI of 100 was determined by lysing THP-1 cells with double distilled water (ddH_2O), then plating on anaerobic blood agar, incubating under anaerobic conditions for 7 days. Each experimental condition was performed independently 9 times. Infection of *E.coli* ATCC 25922 to the THP-1 cell was performed in a similar manner and used as a control to confirm the phagocytic ability of THP-1. The viability of THP-1 cells was monitored by staining with trypan blue.

Inhibition of autophagosome formation

The numbers of intracellular *P.gingivalis* were quantitated at various times in the presence or the absence of the autophagosome inhibitor, wortmannin. THP-1 cells were incubated in RPMI 1640 containing 10 nM wortmannin (Sigma) for 60 min at 37°C in 5% CO_2, prior to addition of the bacteria. The treated cells were further incubated for 90 min, centrifuged, and treated with the antibiotics. After centrifugation, the cells were re-suspended in RPMI 1640 containing 10% FBS and incubated at 37°C in 5% CO_2 for 0, 4 and 8 h. At each time point, the survival of *P.gingivalis* in THP-1cells was then determined. Each condition was performed 4 times independently.

Monocyte migration and survival of P.gingivalis in monocytes after migration

The infected THP-1 cells, after treatment with antibiotics, were plated into the upper compartment of a transwell chamber (polycarbonate membrane, 6 well size, pore size 8 μm, Costar Corporation, Cambridge, MA, USA). Monocyte chemoattractant protein-1 (MCP-1) (Sigma) (10 ng/ml) was added to the lower compartment of the transwell. After incubation at 37°C in 5% CO_2 for 2 h, THP-1 cells in the lower compartment were counted in a hematocytometer under a phase contrast microscope. The media was removed by centrifugation at 137×g for 10 min at 4°C. THP-1 cells were washed once with phosphate buffer saline (PBS), then lysed with sterile ddH_2O and plated on anaerobic blood agar. The plates were incubated under anaerobic condition for 7 days and bacterial colonies were counted. Each experimental condition was performed independently 9 times. THP-1 cells cultured in the media without MCP-1 were considered as a negative control, whereas the uninfected cells were used as a positive control.

IL-1β and TNF-α production after invasion of THP-1 cells by P.gingivalis.

IL-1β and TNF-α production were determined using enzyme-linked immunosorbent assays (ELISAs) kits (R&D Systems, Minneapolis, Minn, USA). Each experiment was performed 10 times. The media of uninfected cells were used as a negative control. The positive control was performed in a similar manner to the experimental group, but without antibiotic treatment. Results were expressed as picograms of IL-1β or TNF-α per milliliter of media.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed by one-way ANOVA and Kruskal-Wallis test using SPSS for Windows version 17 (SPSS, Chicago, IL, USA). A *p*-value < 0.05 was considered as statistically significance.

Results

Bacterial localization in THP-1 cell

At MOI of 1:100 and incubation time of 90 min, *P. gingivalis* showed the maximum survival (data not shown) and the viability of THP-1 cells was >90%. Therefore, the condition using MOI 1:100 and incubation time 90 min was used for all subsequent studies. Infected THP-1 cells showed typical and viable phenotypes, such as an irregular nucleus, euchromatin in the middle and heterochromatin along the periphery of the nucleus (Figure 1A.). The nucleolus was also observed to be intact and eccentrically located in the nucleus (Figure 1A.). Several organelles such as mitochondria, rough endoplasmic reticulum and ribosomes were clearly observed in the cytoplasm (Figure 1A.). The cell surfaces of THP-1 cells also showed pseudopodia (Figure 1A, 1B). *P.gingivalis* appeared to aggregate and adhere at THP-1 cell surfaces (Figure 1A, 1B) with electron dense characteristic (Figure 1B).

After treatment with the antibiotics, no *P.gingivalis* was observed outside the THP-1 cell, either associated with the plasma membrane or extracellularly. The bacteria were clearly visible within the cytoplasm and seemed to be localized within 2 areas, either closely surrounded by a thin membranous vacuole, with ground substances and rough endoplasmic reticulum (Figure 1C), or presented in a large vacuole near the cell surface (Figure 1D). Further incubation in RPMI1640 for 4 h, intracellular *P.gingivalis* was surrounded within a closed, thin membrane, with ground substances and rough endoplasmic reticulum (Figure 1E). In this state, the bacteria could not be detected in vacuoles. Other cellular organelles of THP-1 cells, such as mitochondria, rough endoplasmic reticulum, ribosome and nucleus appeared to be normal. The phenotype of THP-1 cells and intracellular *P.gingivalis* after 8 h further incubation were similar to that observed at 4 h incubation (Figure 1F).

Survival of P.gingivalis in THP-1

The survival efficiency of *P.gingivalis* after incubation for 0, 4 and 8 h were 112.22±20.48, 122.22±25.87, and 114.44±15.09 ($x10^2$ CFU per 10^6 THP-1 cells), respectively (Figure 2). No significant difference in survival of intracellular *P.gingivalis* at different incubation times. In contrast to *E.coli*, the colony forming units (CFUs) decreased with longer time of incubation.

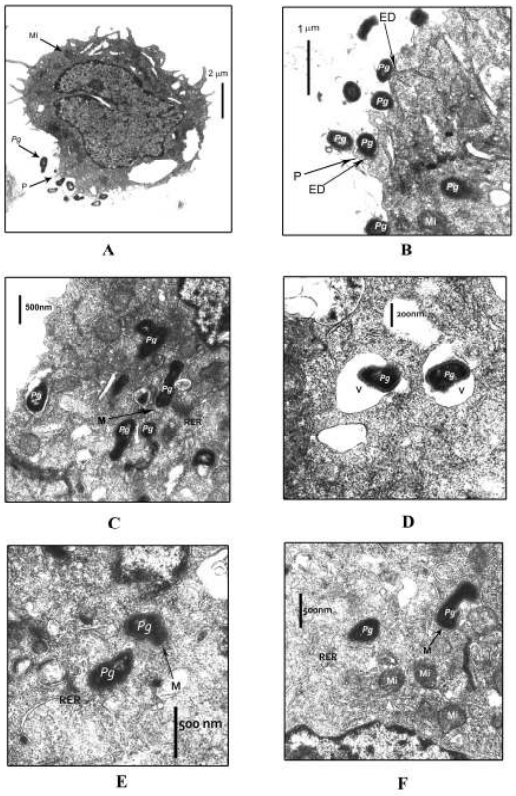

Figure 1. Bacterial localization in the THP-1cells. After infection for 90 min, *P.gingivalis* (*Pg*) were detected at certain areas of the THP-1 cell membrane (Fig. 1A and 1B). At this adhesion area, the cell membrane of the THP-1 was electron dense (ED) and showed pseudopodia (P) (Fig. 1B). After infection and treatment of THP-1 cells with antibiotics, the bacteria were localized in two areas in the cytoplasm: one where the bacteria were surrounded by a thin membranous vacuole (M), cytoplasm, ground substance and rough endoplasmic reticulum (RER) (Fig. 1C), another where bacteria were present in a large vacuole (V) near the cell surface (Fig. 1D). After antibiotics treatment and further incubation in RPMI1640 for 4 h and 8 h, the bacteria were still present in the THP-1 cytoplasm. The bacteria in this state were surrounded by a thin membranous vacuole, cytoplasm, ground substance and rough endoplasmic reticulum (Fig. 1E and 1F).

The CFUs of *E.coli* after 0, 4 and 8 h incubation were 44.22±9.19, 3.33±3.46, and 0±0 CFU ($x10^2$ CFU per 10^6 THP-1 cells), respectively. These results indicate the survival capability of *P.gingivalis* THP-1 cells for at least 8 h in comparison to *E.coli*.

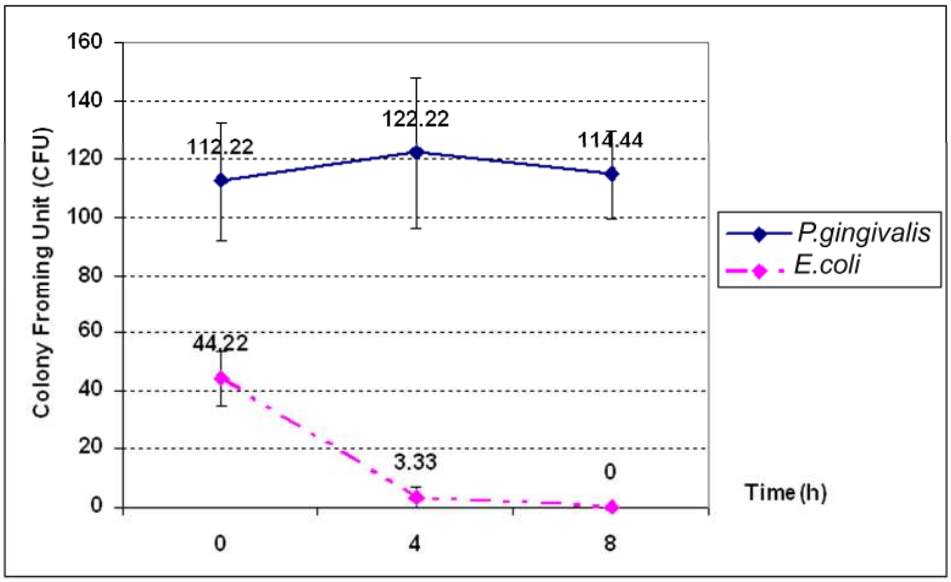

Figure 2. Numbers of colony forming units (x 10^2 CFU per 10^6 cells) of *P.gingivalis* and *E. coli.* The CFU number was counted from colonies of *P.gingivalis* or *E.coli* on blood agar after bacteria were inoculated and incubated with THP-1 cells for 0, 4, 8 h. The CFU of *P.gingivalis* in THP-1 cells did not differ significantly at different incubation times ($P<0.05$) (n=9). In contrast to *P. gingivalis*, *E. coli* CFU differed significantly at different incubation times ($P<0.05$) (n=9).

Inhibitor of autophagosome formation of THP-1 cells

The effect of the phosphatidylinosotol-3 kinase (PI 3-kinase) inhibitor wortmannin, known to inhibit autophagosome formation, on the survival of *P.gingivalis* was also studied. When THP-1 cells were treated with wortmannin before infecting with *P.gingivalis,* CFUs after incubation for 0, 4 and 8 h were much reduced at 45.75±2.99, 40.75±4.65, and 40.00±1.41 (x10^2 CFU per 10^6 THP-1 cells), respectively (Table 1). These indicated that the inhibition of autophagosome affected the survival of *P.gingivalis*.

Monocyte migration

The ability of THP-1 cells to migrate after bacterial infection was also studied using MCP-1 as the chemo-attractant. The mean number of infected THP-1 cells migrating was 18.64±2.33x10^4 cells, while that of uninfected THP-1 cells migrating was 19.11±1.76x10^4 cells (Table 2). These values are much greater than the number of cells migrating in the absence of MCP-1 (1.25±0.57x10^4 cells). There was no significant difference between the number of infected and non-infected THP-1 cells migrating in response to MCP-1. In addition, the mean number of intracellular *P.gingivalis* following the migration stimulated by MCP-1 was comparable to that in non-stimulated THP-1 with lower number of migration.

Production of IL-1β and TNF-α by THP-1 cells after infected by P.gingivalis

As shown in Table 3, the normal level of secreted IL-1β protein from THP-1 cells was 2.9±2.8 pg/ml. THP-1 cells infected with *P.gingivalis* without antibiotics treatment showed significant production of IL-1β with a mean concentration of 186.4±6.6 pg/ml. After antibiotic treatment, a comparable concentration of IL-1β was detected. When the cells were further incubated for 24 h, the mean concentration of IL-1β was significantly increased to 224.4±10.0 pg/ml.

In the absence of *P.gingivalis*, the mean concentration of TNF-α produced by THP-1 was 2.7±2.6 pg/ml. The cytokine level was increased to 881.7±48.9 pg/ml, when cells were infected with *P.gingivalis*. The similar level of 884.1±35.3 pg/ml was found when the infected THP-1 cells were treated with the antibiotics. TNF-α concentration was significantly reduced to 215.9±34.1 pg/ml after further incubation in RPMI for 24 h as shown in Table 3.

Table 1. Inhibition of autophagosome formation of THP-1 cells (n=4). Number of CFU of *P.gingivalis* within THP-1 cells following lysis of the THP-1 cells (n=4), with and without treatment with wortmannin; *, **, *** Significantly different when comparing treated and untreated groups at the same incubation time (*P<0.05*) (n=4).

Incubation time (h)	Treatment with wortmannin (x 10^2 CFU per 10^6 cells) (Mean±SD)	Non-treatment (x 10^2 CFU per 10^6 cells) (Mean±SD)
0	45.75±2.99*	113.00±9.90
4	40.75±4.65 **	114.75±4.92
8	40.00±1.41***	115.25±8.77

Discussion

P. gingivalis is a major pathogen in adult periodontitis and reported to be associated with the atherosclerotic lesions. One possible pathogenic mechanism for this association might include bacterial invasion, intracellular survival and translocation of circulating host cells. However, until now there is no report on the ability of *P.gingivalis* to survive in white blood cells, and in fact they are typically killed when phagocytosed by neutrophils in the presence or absence of serum[16].

In this study, we demonstrated the survival of *P.gingivalis* in THP-1 monocytes. The optimal MOI for *P.gingivalis* was 100, a value similar to that found for other cells, such as gingival epithelial cells[4] and human coronary artery cells[5]. The number of intracellular of bacteria was not increased at higher MOI of 500 and 1000. It is possible that THP-1 cells may have specific surface receptors for *P.gingivalis*, and once these receptors are fully occupied by *P.gingivalis*, increasing number of infecting bacteria will not increase the number of intracellular bacteria. Similarly, optimum incubation time of 90 min is sufficient for the bacteria to bind and enter THP-1 cells; increasing time will not increase the numbers of intracellular bacteria. The attachment and invasion of these bacteria may be initiated by attachment of *P.gingivalis* to THP-1 cell surfaces using specific fimbrillin. Indeed, fimbriae of *P.gingivalis* are a well known virulence factor involved in the adhesion and trafficking process of these bacteria in many mammalian cell types, including epithelial cells[17], endothelial cells[18]and dendritic cells.[19]

TEM studies showed that *P.gingivalis* could invade THP-1 cells and appeared intact within the autophagosome-like structure of THP-1 cells. After 90-min incubation, some bacteria attached to the cell surfaces at the pseudopodia-like structures. Interestingly, most extracellular bacteria appeared to concentrate and adhere at certain areas on the cell membrane. This might be due to bacterial aggregation resulting in the engulfing of aggregated bacteria by the THP-1 cell. The electron density at the adhesion areas suggested the possibility that the THP-1 cell may have specific opsonic receptors for *P.gingivalis* in the certain regions of the cell membrane, similar to that observed in coronary artery cells.[5]

Two forms of phagosomal structures were observed; first, a thin membranous vacuole, autophagosome-like structure, closely surrounding the intracellular bacteria, another form was large vacuoles near the cell surface with bacteria inside. The bacteria were still present in autophagosome after antibiotics treatment and further incubation for up to 8 h. When THP-1 cells were treated with autophagosome inhibitor, wortmannin, intracellular *P.gingivalis* were significantly decreased when compared with the untreated THP-1 cells. This suggests that autophagosome might be the survival site for *P.gingivalis* in THP-1 cells.

Table 2. Number of THP-1 cells in lower compartment of transwell and CFU of *P.gingivalis* per THP-1 cell following lysis of the THP-1 cells (n=9), with and without stimulation by MCP-1; * Significantly different when comparing positive control and experimental group (*P<0.05*) (n=9).

Group	Number of THP-1 cells (×10^4) (Mean±SD)	CFU per THP-1 cell (×10^{-3}) (Mean±SD)
Positive control (MCP-1 stimulated, non-infected THP-1)	19.11±1.76	0
Negative control (Un-stimulated, infected THP-1)	1.25±0.57 *	6.34±2.24
Experimental (MCP-1 stimulated, infected THP-1)	18.64±2.33	5.83±1.45

Table 3. TNF-α and IL-1β production by THP-1 cells after infection with *P.gingivalis* (n=10); * Significantly decreased when compared to infected cells and infected cells treated with antibiotics (*P<0.05*); ** Significantly increased when compared to infected cells and infected cells treated with antibiotics (*P<0.05*) (n=10).

Treatment	Mean ± SD (pg/ml)	
	TNF-α	IL-1β
Non-infected cells	2.7±2.6	2.9±2.8
Infected cells	881.7±48.9	186.4±6.6
Infected cells and treated with antibiotics	884.1±35.3	189.5±6.0
Infected cells and treated with antibiotics and RPMI	215.9±34.1*	224.4±10.0**

The doubling time of *P.gingivalis* in media under strictly anaerobic condition was found to be about 4 h. In human coronary artery cells, intracellular *P.gingivalis* increased 2.7 folds after 5.5 h incubation.[20] However in the present study, the number of *P.gingivalis* did not change up to 8 h. In contrast to *E.* coli, the bacteria were all killed after 8 h incubation. This suggested that *P.gingivalis* may have specific mechanism to escape from the killing process of THP-1 cells. Together with the TEM study, we believed that *P.gingivalis* can survive in monocytes within the autophagosome-like structures. Whether the bacteria can replicate or not, required longer time of incubation. The autophagocytic pathway is an important cellular pathway for the selective degradation of nonfunctional cellular organelles and proteins in order to maintain cell functions. [21, 22] Normally, phagocytosed extracellular bacteria cannot traffic to the autophagosomes. However, *P.gingivalis* may have specific trafficking pathway to autophagosomes, which allows them to escape from the intracellular killing mechanism of the phago-lysosomes. The use of autophagosomes as a survival site within the host cell is also found in *Brucellar abortus*[23] and *Legionella pneumophila*.[24] Other mechanisms for the survival of bacteria in autophagosomes involve the secretion of short peptides and proteins which can inhibit lysosomal enzymes.[20] The trafficking pathway to autophagosomes of *P.gingivalis* in THP-1 need to be elucidated. This ability has also been reported in *P.gingivalis*-infected endothelial cells.[20] In other cells, including gingival epithelium cells[4], and human junctional epithelium cells.[25] *P.gingivalis* was found to be free in the cytoplasm. The finding of *P.gingivalis* within the endosomes in oral epithelial cells[3], suggested another mechanism that the sulfatides synthesized by *P.gingivalis* may prevent the fusion of phagosome and lysosome in a similar manner to action of sulfatides[26] or lipid phosphatase[27] produced by *Mycobacterium tuberculosis* which is a macrophage-specific intracellular pathogen. Thus, *P.gingivalis* might have the specific survival mechanisms within particular host cells.

Results from trans-membrane migration of infected THP-1 cells suggested that intracellular *P.gingivalis* did not affect the migration ability of THP-1 cells. In addition, MCP-1, a chemotactic cytokine inducing monocyte migration by activating PI 3-kinase[11], is required in autophagosome formation.[28] In the presence MCP-1, the number of survival *P.gingivalis* per cell was unchanged. The migration and stimulation of monocytes with MCP-1 did not affect the survival ability of intracellular *P.gingivalis*.

The monocytes have many important functions in host responses against bacterial infection, including secretion of cytokines such as TNF-α and IL-1β. Results from ELISA studies indicated that intracellular *P.gingivalis* could stimulate IL-1β and TNF-α production of THP-1 cells, similar to the stimulation by extracellular bacteria[19] and lipopolysaccharides (LPS).[29] IL-1β and TNF-α mRNA expression were also detected in infected THP-1 cells by reverse transcription-polymerase chain reaction (RT-PCR) technique (data not shown), confirming the cytokine production results. The mean concentration of IL-1β was significantly increased after stimulating THP-1 cells with *P.gingivalis*, and followed by treatment with the antibiotics and further incubation for 24 h. On the other hand, the mean concentration of TNF-α produced by THP-1 cells after similar treatment was significantly decreased. This may be due to the different characteristics of cell stimulation by IL-1β and TNF-α. In addition, IL-1β stimulated cells can be re-stimulated resulting in the up-regulation of IL-1β, while TNF-α stimulated cells cannot. These results supported the idea that IL-1β is a potent pro-inflammatory cytokine in humans, which continues to be

produced for a long period of time after stimulation.[9]

Our study provides an explanation for the occurrence of atherosclerotic lesion by *P.gingivalis*, in which monocytes may be used as a vehicle for transporting *P.gingivalis* along the blood circulation to the adhesion sites. The atherosclerotic lesion was progressed by stimulating the production of pro-inflammatory cytokines. However, the study in animal models would be required to confirm the *in vitro* observations.

Acknowledgements

We are very grateful to Prof. M.R. Jisnuson Svasti for English correction and Assoc. Prof. Kleophanth Thakerngpol for TEM facility. This study was fully supported by the Royal Golden Jubilee Grant from Thailand Research Fund.

References

1. Slots J, Ting M. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. Periodontol 2000 1999; 20: 82-121.
2. Rudney JD, Chen R, Sedgewick GJ. Intracellular *Actinobacillus actinomycetemcomitants* and *Porphyromonas gingivalis* in buccal epithelial cells collected from human subjects. Infect Immun 2001; 69: 2700-7.
3. Sandros J, Papapanou P, Dahlen G. *Porphyromonas gingivalis* invades oral epithelial cells in vitro. J Periodontal Res 1993; 28: 219-26.
4. Lamont RJ, Chan A, Belton CM, *et al.* *Porphyromonas gingivalis* invasion of gingival epithelial cells. Infect Immune 1995; 63: 3878-85.
5. Dorn BR, Dunn WA, Progulske-Fox A. Invasion of human coronary artery cells by periodontal pathogens. Infect Immun 1999; 65: 5792-8.
6. Beck JD, Eke P, Lin D, *et al*. Associations between IgG antibody to oral organisms and carotid intima-medial thickness in community-dwelling adults. Atherosclerosis 2005; 183: 342-8.
7. Li L, Messas E, Batista EL Jr, *et al*. Infection accelerates the progression of atherosclerosis in heterozygous apolipoprotein E-deficient murine model. Circulation 2002; 105: 861-7.
8. Brodala N, Merricks EP, Bellinger DA, *et al*. *Porphyromonas gingivalis* bacteremia induces coronary and aortic atherosclerosis in normocholesterolemic and hypercholesterolemic pigs. Arterioscler Thromb Vasc Biol 2005; 25: 1446-51.
9. Kjeldsen M, Holmstrup P, Bendtzen K. Marginal periodontitis and cytokines: A review of the literature. J Periodontol 1993; 64: 1013-22.
10. Ozeren A, Aydin M, Tokac M, *et al.* Levels of serum IL-1β, IL-2, IL-8 and tumor necrosis factor-α in patients with unstable angina pectoris. Mediators Inflamm 2003; 12: 361-5.
11. Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. Circ Res 2004; 95: 858-66.
12. Qi M, Miyakawa H, Kuramitsu HK. *Porphyromonas gingivalis* induces murine macrophage foam cell formation. Microb Pathog 2003; 35: 259-67.
13. Carroll GC, Sebor RJ. Dental flossing and its relationship to transient bacteremia. J Periodontol 1980; 51: 691-2.
14. Lockhart PB, Brennan MT, Sasser HC, *et al.* Bacteremia associated with toothbrushing and dental extraction. Circulation 2008; 117: 3118-25.
15. Silver JG, Martin AW, McBride BC. Experimental transient bacteraemias in human subjects with varying degrees of plaque accumulation and gingival inflammation. J Clin Periodontol 1977; 4: 92-9.
16. Cutler CW, Kalmar JR, Arnold RR. Phagocytosis of virulent *Porphyromonas gingivalis* by human polymorphonuclear leukocytes requires specific immunoglobulin G. Infect Immun 1991; 59: 2097-104.
17. Weinberg A, Belton C, Park Y, *et al.* Role of fimbriae in *Porphyromonas gingivalis* invasion of gingival epithelium cells. Infect Immun 1997; 65: 313-6.
18. Deshpande RG, Khan MB, Genco CA. Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*. Infect Immun 1998; 66: 5337-43.
19. Jotwani R, Cutler CW. Fimbriated *Porphyromonas gingivalis* is more efficient than fimbria-deficient *P.gingivalis* in entering human dendritic cells in vitro and induces an inflammatory Th1 effector response. Infect Immun 2004; 72: 1725-32.
20. Dorn BR, Dunn WA, Progulske-Fox A. *Porphyromonas gingivalis* traffics to autophagosomes in human coronary artery endothelial cells. Infect Immun 2001; 69: 5698-708.
21. Mortimore GE, Poso AR, Lardeux BR. Mechanism and regulation of protein degradation in liver. Diabetes Metab Rev 1989; 5: 49–70.
22. Luzikov VN. Quality control: from molecules to organelles. FEBS Lett 1999; 448: 201–5.
23. Pizarro-Cerdá J, Méresse S, Parton RG, *et al.* *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect Immun 1998; 66: 5711–24.
24. Brand BC, Sadosky AB, Shuman HA. The *Legionella pneumophila* icm locus: a set of genes required for intracellular multiplication in human macrophages. Mol Microbiol 1994; 14: 797-808.
25. Papapanou PN, Sandros J, Lindberg K, *et al*. *Porphyromonas gingivalis* may multiply and advance within stratified human junctional epithelium *in vitro*. J Periodont Res 1994; 29: 374-5.
26. Goren MB, Hart PD, Young MR, *et al*. Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of *Mycobacterium tuberculosis*. Proc. Nat. Acad. Sci. USA 1976; 73: 2510-14.
27. Vergne I, Chua J, Lee HH, *et al.* Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. Proc. Nat. Acad. Sci. USA 2005; 102: 4033-38.
28. Blommaart EFC, Krause U, Schellens JPM, *et al.* The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur J Biochem 1997; 243: 240-6.
29. Baqui AAMA, Meiller TF, Chon JJ, *et al*. Granulocyte-Macrophage colony-stimulating factor amplification of interleukin-1β and tumor necrosis factor alpha production in THP-1 human monocytic cells stimulated with lipopolysaccharide of oral microorganisms. Clin Diagn Lab Immunol 1998; 5: 341-7.