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Treponema denticola induces interleukin-8 and macrophage chemoattractant
protein 1 production in human umbilical vein epithelial cells

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Abstract

Treponema denticola, a major pathogen of periodontitis, has also been detected in the lesions of atherosclerosis. The aim of this study was to investigate induction of chemokine production in human umbilical vein endothelial cells (HUVECs) by *T. denticola* and determine whether those chemokines were degraded by a protease, dentilisin. *T. denticola* ATCC35405 or dentilisin-deficient mutant K1 were added to HUVECs and levels of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) in the culture supernatants were determined by enzyme-linked immunosorbent assay. *T. denticola* ATCC35405 induced production of IL-8 in a time-dependent manner, with both production of IL-8 and expression of IL-8 mRNA showing higher levels than with exposure to dentilisin-deficient mutant K1. Although exposure to ATCC35405 induced expression of MCP-1 mRNA in the HUVECs, MCP-1 levels were remained similar to that in unstimulated cells. IL-8 and MCP-1 showed partial hydrolysis with exposure to *T. denticola* ATCC35405, but not with *T. denticola* K1. These results suggest that *T. denticola* can evade host defense mechanisms by modulating production of IL-8 and MCP-1, and that this play a role in the development of chronic infections such as periodontitis. The association of *T. denticola* infection to atherosclerosis was also discussed based on the present study.

Key word: *Treponema denticola*, Atherosclerosis, Chemokine, Periodontitis, Protease

1. Introduction

Periodontal disease is an inflammatory disease caused by Gram-negative rods and spirochetes [1]. Recent epidemiological and experimental studies have revealed an association between periodontal disease and vascular disease [2-4]. Activation of endothelial cells and leukocyte-endothelial interaction play a central role in inflammation [5]. Vascular endothelial cells respond to environmental stimuli by recruiting host defense blood cells via expression of adhesion molecules and chemotactic cytokines involved in inflammation [6]. The role of endothelial cells in the progression of periodontitis remains to be fully clarified, although they are a major cell in highly-vascularized periodontal tissue.

One of the most potent chemoattractants of monocytes is monocyte chemoattractant protein-1 (MCP-1), a C-C chemokine, and monocyte activation by MCP-1 derived from endothelial cells is the first step in monocyte infiltration of inflamed tissue [7]. Interleukin 8 (IL-8), a chemokine, specifically directs polymorphonuclear cell migration [8]. Infiltration by mononuclear cells is a major event in chronic inflammation such as that found in periodontitis and atherosclerosis. Levels of IL-8 and MCP-1 were reported to show an increase in gingival crevicular fluid (GCF) [9-11]. It has been demonstrated that

MCP-1 is synthesized in inflamed gingiva by vascular endothelial cells and mononuclear phagocytes [12]. Apostolopoulos et al. [13] found IL-8 mRNA in carotid plaque, and Moreau et al. [14] showed that IL-8 played a potential role in atherogenesis by inhibiting tissue inhibitors of metalloproteinase-1 expression. MCP-1 has been suggested to induce macrophage infiltration into the arterial wall [15].

T. denticola has frequently been isolated from lesions resulting from chronic periodontitis and acute ulcerative necrotizing gingivitis [16, 17]. It has also been detected in lesions of vascular disease such as atherosclerosis and Buerger's disease [18, 19]. This microorganism has several virulence factors, including prolylphenylalanine-specific protease dentilisin, a major outer sheath protein [17]. Dentilisin has been shown to be involved in the modulation of the inflammation process through inactivation of interleukin-1 β , IL-6 and tumor necrosis factor α (TNF α) by degradation [20] and activation of complement by cleaving C3 [21]. Whether *T. denticola* can induce chemokines remains to be determined, although it has been isolated from lesions in which chemokines were found.

The purpose of this study was to investigate the ability of *T. denticola* to induce IL-8 and MCP-1 production, to clarify the role of dentilisin on these mediators to clarify the involvement of this microorganism in the pathogenesis of periodontitis and to find the

association to atherosclerosis.

2. Materials and Methods

2.1 Bacterial Strains and Culture Conditions

T. denticola ATCC 35405 and dentilisin-deficient mutant K1 [22] were used in this study. Microorganisms were grown in TYGVS medium under anaerobic conditions at 37°C for 72 h [23]. To maintain *T. denticola* K1, 10 µg/ml erythromycin was added to the medium. Bacterial numbers were estimated by measuring optical density (OD) at 600 nm using a spectrophotometer UV-2550 (Shimazu, Kyoto, Japan).

2.2 Culture of HUVECs

Primary cultured HUVECs were obtained from Cascade Biology (Portland, OR). The cells were cultured in Humedia-EG2 medium (KURABO, Osaka, Japan) in a collagen-coated 12-well polystyrene plate (Ashahi Techno Glass Corporation, Tokyo, Japan) in a humidified chamber with 5% CO₂ in air at 37°C. After treatment with Trypsin-EDTA (Invitrogen/Gibco, Carlsbad, CA), the cells were counted with the ZI Series Coulter Counter (Beckman Coulter, Fullerton, CA) and processed for cytokine production assay.

2.3 Evaluation of cytokine production in HUVECs by coincubation with *T. denticola*

The HUVECs were cultured in the 12-well plate in endothelial cell medium (Kurabo Industries LTD, Osaka, Japan). After the cells were grown to confluency, the medium was removed and fresh medium containing *T. denticola* or medium alone were added to a final volume of 2 ml and incubated for 6 h, 9 h, 16 h and 24 h. In the preliminary trials, *T. denticola* showed its highest rate of adherence and invasion in the HUVECs at a multiplicity of infection (MOI) rate of 100, and this was the MOI used in this study. Cells without exposure to *T. denticola* were used as a control. After incubation, concentrations of IL-8 and MCP-1 in the culture medium were quantified using an enzyme-linked immunosorbent assay kit (ELISA kit, Endogen, Milwaukee, WI) according to the manufacturer's instructions.

2.4 Quantification of mRNA expression of IL-8 and MCP-1 by RT-PCR

After exposure to *T. denticola*, the HUVECs were removed from the plate by trypsinization. Total RNA was isolated from 10^5 HUVECs with an RNA extraction kit (Qiagen, Valencia, CA), and cDNA was synthesized by Omniscript reverse transcriptase (Qiagen) from about 90 ng of obtained total RNA using oligo-dT primer (Invitrogen, Carlsbad, CA) and Random decamers (Ambion, Ausitin, TX) according to manufacturer's protocol. For real-time PCR, primers and Taqman probes for IL-8 (Hs001714103_m1), MCP-1 (Hs00234140_m1) and

GAPDH (Hs99999905_m1) were obtained from Applied Biosystems (Foster City, CA).

Quantitative real-time PCR was performed using the Applied Biosystems Prism 7700

Sequence Detection System (Applied Biosystems) according to the manufacturer's

instructions. Results were expressed as a fold modulation over the expression of GAPDH as

an internal control [20]. Cells without exposure to *T. denticola* were used as a control.

2.5 Degradation of IL-8 and MCP-1 by *T. denticola* protease

Recombinant human IL-8 (1000 pg/ml: R&D Systems, Inc., Minneapolis, MN) and MCP-1 (2000 pg/ml: R&D Systems, Inc.) were incubated with *T. denticola* ATCC 35405 (10^7 cells/ml), K1 (10^7 cells/ml) and 50 mM. phenylmethylsulfonyl fluoride- (PMSF) treated *T. denticola* ATCC 35405 in PBS at 37°C for 12 h in PBS. PMSF was used to inactivate dentilisin. After incubation, concentrations of IL-8 and MCP-1 in the mixture were measured with the ELISA kit (Endogen) as described above. Reduction in IL-8 and MCP-1 after exposure to *T. denticola* was expressed as a percentage of each cytokine level incubated in PBS without *T. denticola*.

2.6 Statistical analysis

To obtain multiple comparisons of cytokine production, statistical significance was determined with an ANOVA followed by the Student-Newman-Keuls test. Comparison of

cytokine mRNA expression in two groups was performed with the Mann-Whitney U test.

3. Results

3.1 Determination of MOI

To determine the levels of IL-8 and MCP-1 induced by *T. denticola*, we cocultured the HUVECs with *T. denticola* at a MOI of 10, 100 and 1000 for 16 h, and performed an ELISA. In our preliminary studies, we found that the number of *T. denticola* cells which adhered to the HUVECs increased depending on the MOI, reaching a plateau at a MOI of 100 (data not shown). When the HUVECs were exposed to *T. denticola* at a MOI of 1000, about 70% of the HUVECs detached from the plate. Results of the viability assay by release of lactose dehydrogenase indicated that about 45% of the HUVECs died (data not shown). Based on these results, evaluation of cytokines was performed at a MOI of 100.

3.2 Production and mRNA expression of IL-8 and MCP-1 in HUVECs by *T. denticola*

IL-8 production was evaluated after exposure of the HUVECs to *T. denticola* ATCC 35405 at a MOI of 100 for 6 h, 9 h, 16 h and 24 h. As shown in Fig. 1, IL-8 levels were significantly higher than those in uninfected cells, suggesting that *T. denticola* induces production of IL-8. We confirmed IL-8 level increased in a time-dependent manner. Other research groups [24, 25] found that cytokine levels in uninfected HUVECs were high,

suggesting that in vitro conditions themselves are responsible for high cytokine base lines.

Taking this into consideration, we believe that the IL-8 assay used in this study was adequate.

The protein levels of MCP-1 in the culture supernatant after exposure to *T. denticola* ATCC 35405 at 6 h, 9 h, 16 h and 24 h are shown in Fig. 2. Unexpectedly, we found no statistically significant difference in MCP-1 production in the culture supernatants between that with exposure to *T. denticola* ATCC 35405 and that without exposure.

Fig. 3 shows the protein and mRNA expression levels of IL-8 in the HUVECs exposed to the *T. denticola* ATCC 35405 and K1 (dentilisin-deficient mutant) strains. It was found that IL-8 protein level in the supernatant after exposure to *T. denticola* ATCC 35405 for 24 h was significantly higher than that with exposure to K1, as shown in Fig. 3 A ($P < 0.05$). IL-8 mRNA expression level in the HUVECs after exposure to *T. denticola* ATCC 35405 was significantly higher than that with K1, as shown in Fig. 3 B ($P < 0.01$).

Fig. 4 shows MCP-1 concentration in the culture supernatant and mRNA expression level in the HUVECs after exposure to *T. denticola* ATCC 35405 and K1. It was found that MCP-1 concentration in the culture supernatant of the HUVECs after exposure to *T. denticola* K1 for 24 h was significantly higher than that with ATCC 35405. MCP-1 mRNA expression level in the HUVECs after exposure to the ATCC 35405 was similar to that with K1.

3.3 Degradation of IL-8 and MCP-1 by *T. denticola* protease

We examined recombinant IL-8 and MCP-1 degradation by *T. denticola* protease.

Quantities of IL-8 and MCP-1 were evaluated by ELISA. After incubation with *T. denticola*

ATCC 35405, about 30% of the IL-8 (Fig. 5) and 99% of the MCP-1 were degraded (Fig. 6).

However, IL-8 and MCP-1 were not reduced by K1 or PMSF-treated ATCC 35405.

4. Discussion

The present study found that exposure of HUVECs to wild-type *T. denticola* or protease-deficient mutant K1 cells initially induced production of IL-8 and MCP-1, with subsequent degradation of MCP-1 with exposure to *T. denticola* wild type. We used uninfected primary HUVECs, as we intended to investigate the ability of *T. denticola* to activate endothelial cells. This stimulation of IL-8 production suggests that *T. denticola* contribute to systemic levels of this chemokines.

IL-8 and MCP-1 are potent chemokines, directing neutrophils and monocytes to sites of infection, and they have been reported to show an increase in gingival crevicular fluid (GCF) from periodontal lesions [9, 11]. Asai et al. [26] reported that *T. denticola* induced IL-8 mRNA in human gingival epithelial cells via Toll-like receptor 2. In our preliminary results,

stimulation of HUVECs for 6 h with 1 µg/ml LPS, an agonist of Toll-like receptors, resulted in a 4.2 times higher increase in induction of IL-8 (data not shown). It is possible that the induction of IL-8 with exposure of HUVECs to *T. denticola* was mediated via Toll-like receptors.

On the other hand, MCP-1 levels in the culture supernatant of the HUVECs exposed to ATCC 35405 were statistically lower than those with exposure to the K1 strain. This finding agrees with that of a previous report which found that MCP-1 production was inhibited by *T. denticola* [27]. Nixon et al. [28] also found that MCP-1 induction by *T. denticola* was low compared to that with *Treponema pectinovorum*. In the present study, no distinct difference was found in level of MCP-1 mRNA expression between ATCC 35405 and K1. However, cytokine levels in the culture supernatant were significantly lower with ATCC 35405 than with K1. These results suggest that MCP-1 is degraded by dentilisin.

We found degradation of IL-8 by ATCC35405, but not by dentilisin-deficient K1, suggesting that dentilisin degrades IL-8. Previous studies have found that exposure to *T. denticola* did not result in production of IL-8 in human gingival epithelial cells or KB cells by ELISA [26, 29]. Asai et al. [26] reported that IL-8 produced from gingival epithelial cells was hydrolyzed by the proteolytic activity of *T. denticola*. These studies did show, however,

IL-8 mRNA expression following exposure to *T. denticola* ATCC 35405, but with degradation of IL-8. This appears to support our finding suggesting that IL-8 is degraded by dentilisin.

In the present study we found that *T. denticola* ATCC 35405 induced significantly higher IL-8 production than K1. However, there was also evidence that ATCC 35405 strain degraded IL-8. IL-8 mRNA expression level was also higher in ATCC 35405-exposed cells than that in K1-exposed cells. One study has shown that surface structures of *T. denticola* were subject to pleiotropic effects due to a deficiency of dentilisin [22]. The results of this study showed that either dentilisin or some other surface structure of *T. denticola* wild type induced IL-8 from HUVECs more strongly than the surface structure of the dentilisin-deficient mutant. However, further analysis is required to determine exactly which surface structure is involved.

The recruitment and adhesion of circulating leukocytes to endothelial cells are early steps in the inflammatory response characteristic of chronic inflammation. *T. denticola* has been reported to activate immune cells and induce cytokines and complement [30, 31].

Periodontopathic bacteria such as *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* induced MCP-1 and IL-8 [32]. Our data on *T. denticola* stimulated IL-8 and MCP-1 productions suggests that *T. denticola* modulates systemic levels of these

chemokines. Chemokine-induced infiltration of inflammatory cells may be involved in the development of chronic inflammation such as that seen in periodontitis. On the other hand, this microorganism hydrolyzed MCP-1 and IL-8 in this experiment. Dentilisin also hydrolyzes IL-1 β , IL-6 and TNF α , and is thought to modulate host defenses [20]. Chae et al. [33] reported that susceptibility to interstitial polymicrobial infection was due to impaired monocyte recruitment in MCP-1-deficient mice. It is possible that the ability of dentilisin to degrade MCP-1 and IL-8 around *T. denticola* contributes to colonization and evasion of host defenses by this microorganism.

Herder et al. [34] reported that elevated systemic levels of chemokines MCP-1, IL-8 and interferon- γ -inducible protein-10 preceded coronary heart disease. Adherence of macrophages to arterial endothelial cells following migration of these cells to endothelial areas is a hallmark of early atherogenesis [35]. Clinical studies have noted the occurrence of bacteremia following dental treatments such as tooth extraction, scaling and root planning [36]. Seinost et al.[37] reported that treatment of severe periodontitis reversed endothelial dysfunction. Periodontal pathogens and their products may invade and contaminate the blood stream, thus affecting endothelial cells. It is possible that the chemokine-inducing activity of *T. denticola* may accelerate the formation of atherosclerosis lesions. Many

epidemiological studies have found an association between periodontitis and atherosclerosis [2, 3, 38]. Spahr et al. [39] investigated the relationship between coronary heart disease and antibody response to periodontopathic bacteria. They reported that *T. denticola*, *P. gingivalis* and *Tannerella forsythensis* were significantly related to coronary heart disease in smokers. Our group and others have detected these periodontopathic bacteria in lesions with atherosclerotic plaque and Buerger's disease [18, 40, 41]. We also compared periodontal pathogen detection rates in stenotic coronary artery plaque from patients with four or more periodontal lesions with those in patients with fewer than four lesions. *T. denticola* was detected in coronary artery samples in 2 out of 17 patients with fewer than four periodontal lesions and in 10 out of 34 patients with four or more lesions. [18]. Beck et al. [42] found that the odds ratio of high antibody levels against *T. denticola* was 1.7 (95% confidence interval = 1.2 - 2.3). These findings suggest the involvement of *T. denticola* in the development of atherosclerosis. However, the precise mechanism behind this periodontopathic bacterial involvement remains to be clarified. Further analysis using different types of cell such as oral epithelial cells and human arterial endothelial cells is required to determine the mechanism of this activity.

In the present study, we showed that *T. denticola* stimulated mRNA expression and

protein production of IL-8 and MCP-1 mRNA. On the other hand, *T. denticola* also degraded these mediators. These properties may contribute to the pathogenesis of periodontal disease and atherosclerosis by contributing to initiation of inflammation and evasion of host defense mechanisms.

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Figure legends

Fig. 1 IL-8 production in culture supernatant of HUVECs after exposure to *T. denticola* ATCC 35405. HUVECs were incubated with *T. denticola* ATCC 35405 for 6 h, 9 h, 16 h and 24 h at MOI 100. Experiments were performed in triplicate and repeated three times. $*(P<0.05)$

Statistically significant difference by Mann-Whitney U test

Fig. 2 MCP-1 production in culture supernatant of HUVECs after exposure to *T. denticola* ATCC 35405. HUVECs were incubated with *T. denticola* ATCC 35405 for 6 h, 9 h, 16 h and 24 h at MOI 100. Experiments were performed in triplicate and repeated three times.

Fig. 3 IL-8 concentration in culture supernatant and mRNA expression in HUVECs after exposure to *T. denticola* ATCC 35405 or K1. HUVECs were incubated with *T. denticola* ATCC 35405 for 20 h at MOI 100.

A: IL-8 concentration in culture supernatant of HUVECs exposed to *T. denticola* ATCC 35405 or K1. Experiments were performed in triplicate and repeated three times.

B: mRNA expression level of IL-8 in HUVECs exposed to *T. denticola* ATCC 35405 or K1. Experiments were performed in triplicate and repeated three times.

* $P<0.05$, ** $P<0.01$ by ANOVA followed by Newman-Keuls test

Fig. 4 MCP-1 concentration in culture supernatant and mRNA expression in HUVECs after exposure to *T. denticola* ATCC 35405 or K1. HUVECs were incubated with *T. denticola* ATCC 35405 for 20 h at MOI 100.

A: MCP-1 concentration in culture supernatant of HUVECs exposed to *T. denticola* ATCC 35405 or K1. Experiments were performed in triplicate and repeated three times.

B: mRNA expression level of IL-8 in HUVECs exposed to *T. denticola* ATCC 35405 or K1. Experiments were performed in triplicate and repeated three times.

* $P < 0.05$, ** $P < 0.01$ by ANOVA followed by Newman-Keuls test

Fig. 5 Degradation of IL-8 by *T. denticola*. After recombinant IL-8 was incubated with *T. denticola* (2×10^7) for 12 h, supernatant of reaction mixture was processed for ELISA.

Reduction in IL-8 after exposure to *T. denticola* is expressed as percentage of IL-8 level with incubation in PBS. Experiments were performed in triplicate and repeated three times.

* $P < 0.05$, ** $P < 0.01$ by ANOVA followed by Newman-Keuls test

Fig. 6 Degradation of MCP-1 by *T. denticola*. After recombinant MCP-1 was incubated

with *T. denticola* (2×10^7) for 12 h, supernatant of reaction mixture was processed for ELISA.

Reduction in MCP-1 after exposure to *T. denticola* is expressed as percentage of MCP-1 level

with incubation in PBS. Experiments were performed in triplicate and repeated three times.

* $P < 0.05$, ** $P < 0.01$ by ANOVA followed by Newman-Keuls test

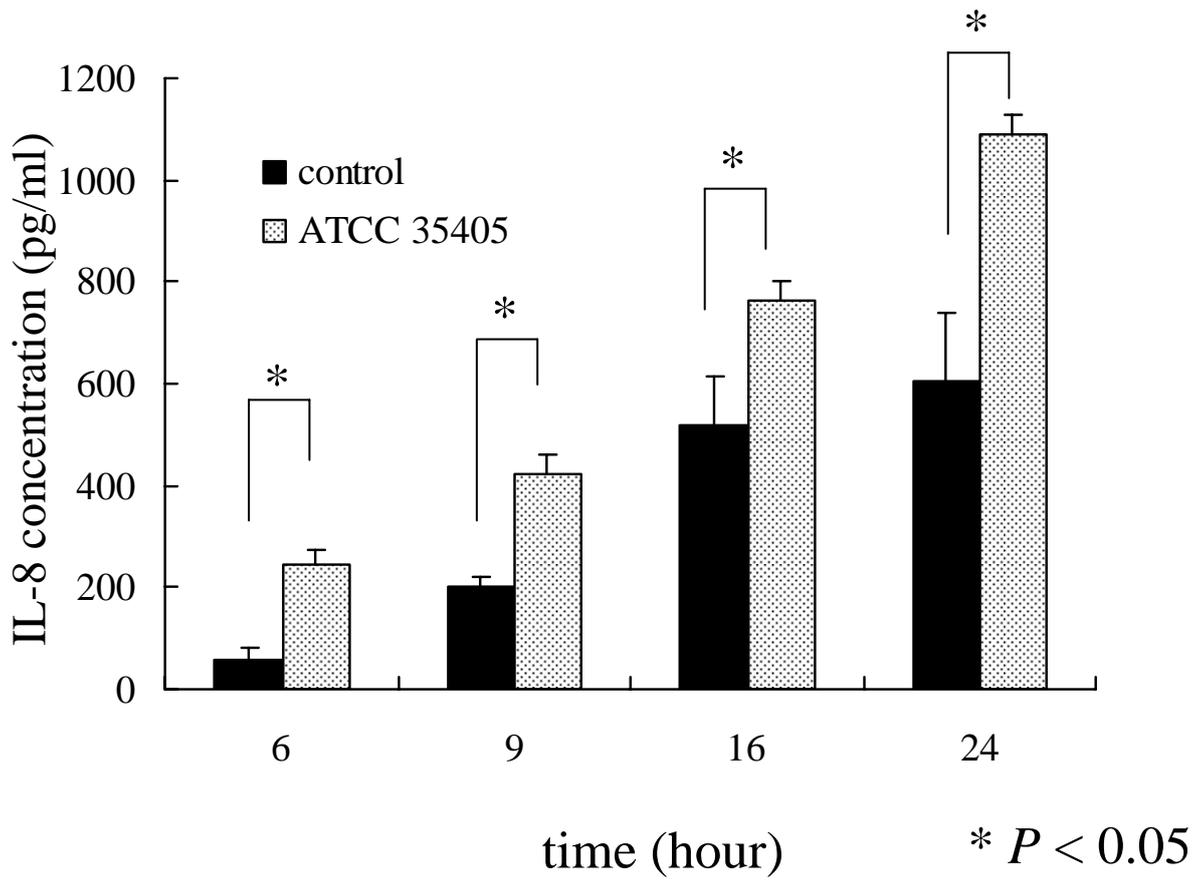


Fig. 1

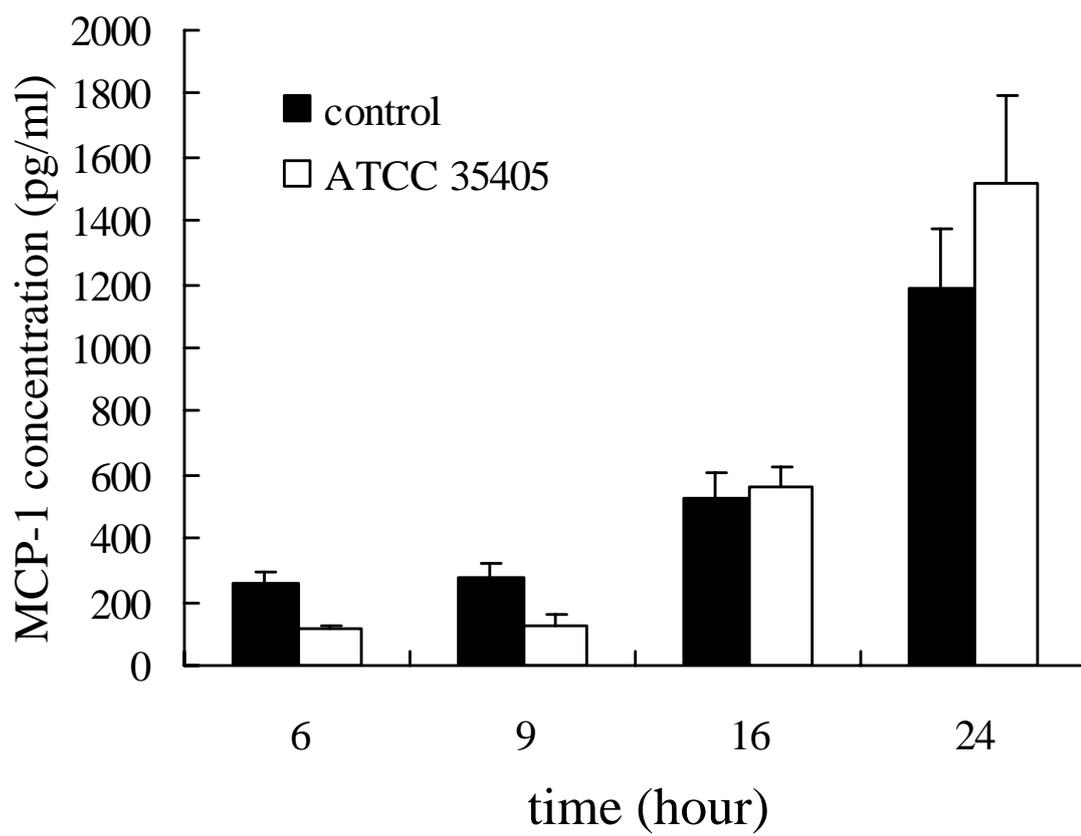


Fig. 2

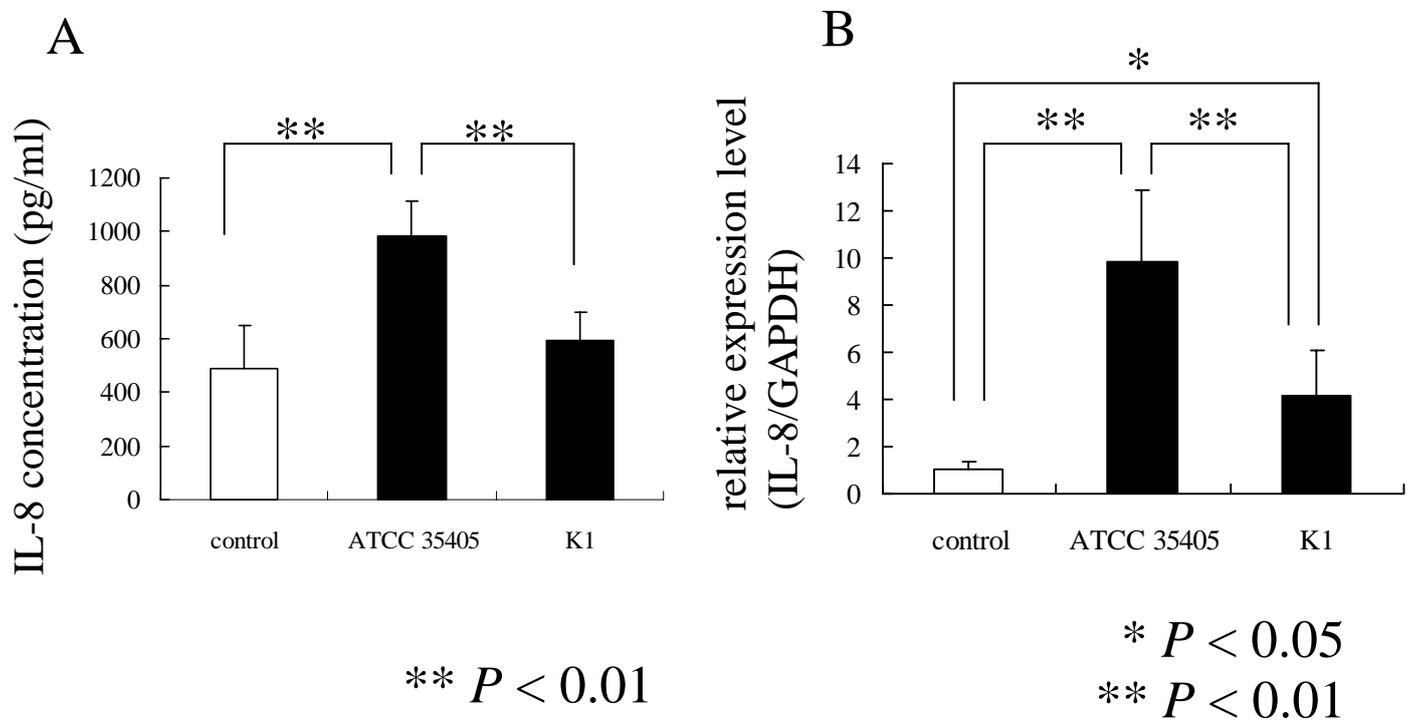
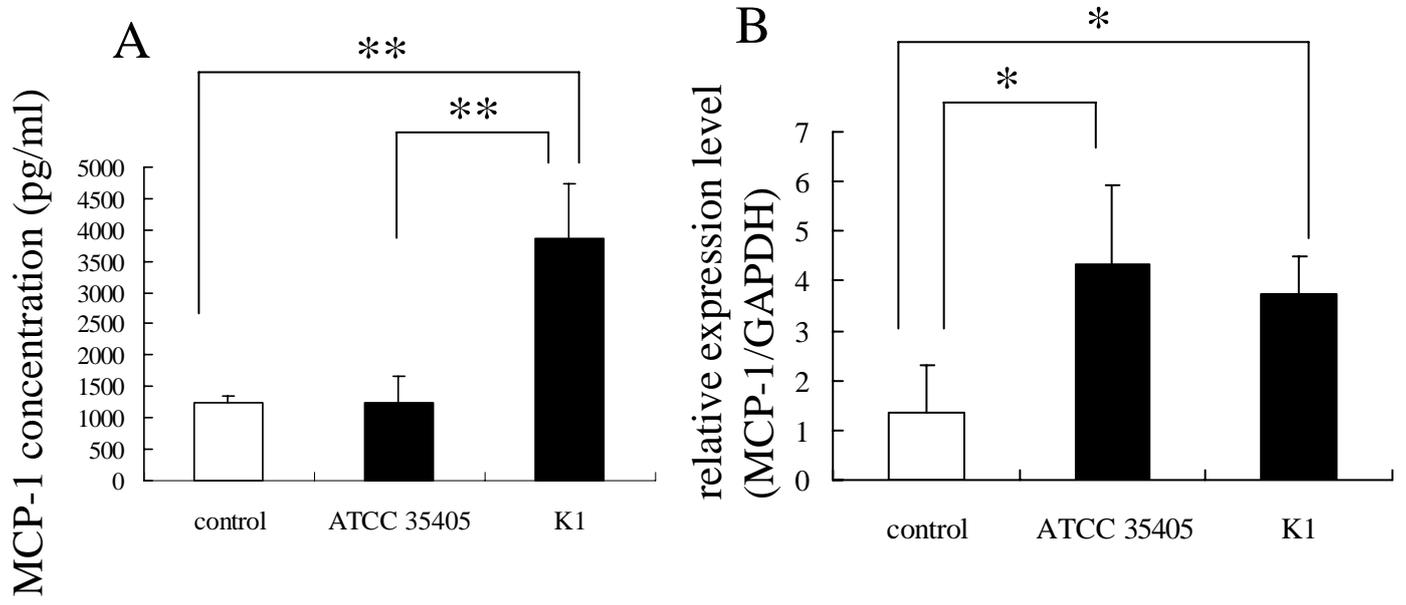


Fig. 3

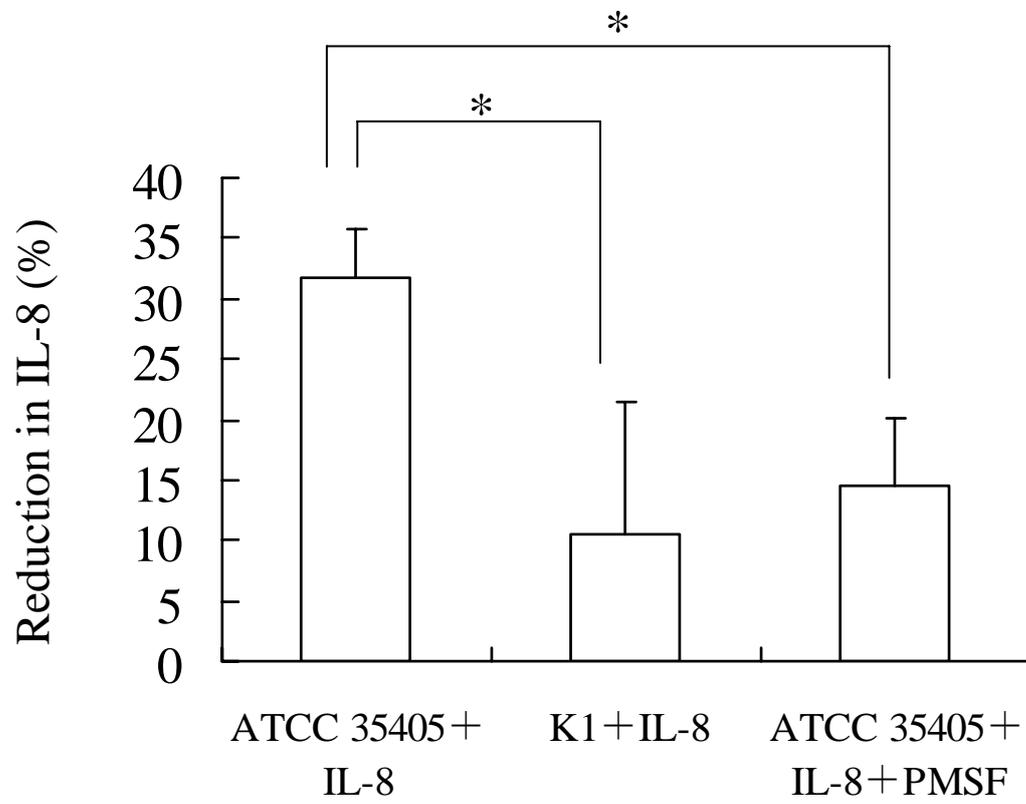


** $P < 0.01$

* $P < 0.05$

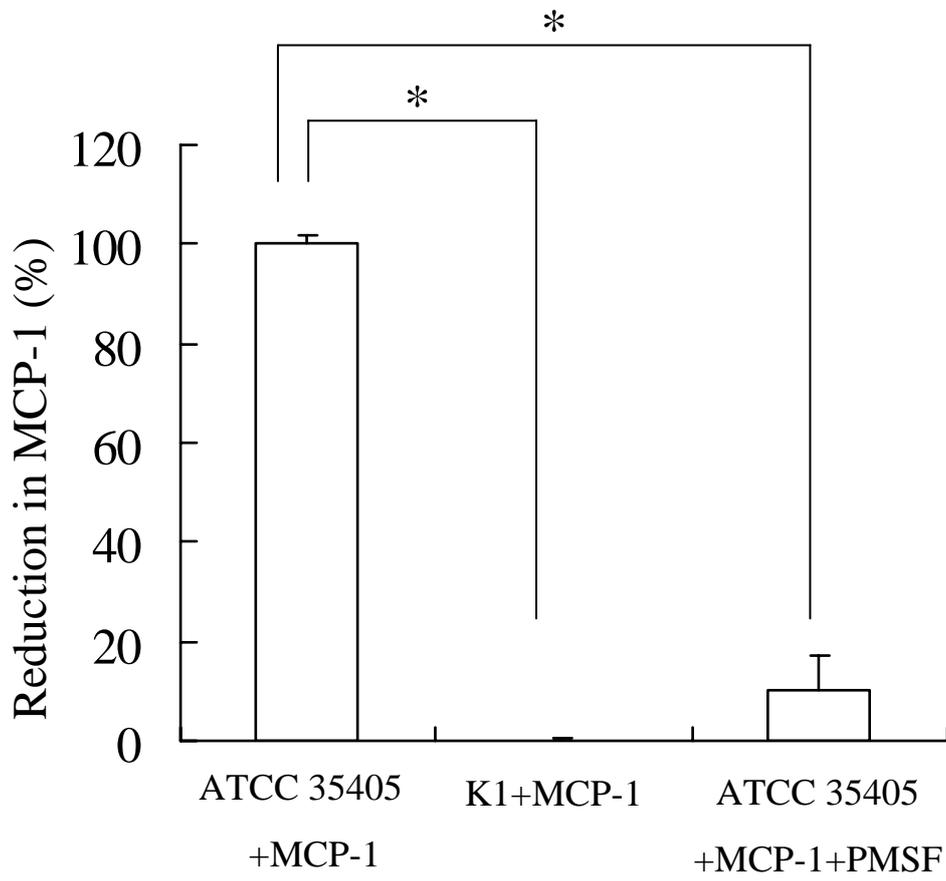
** $P < 0.01$

Fig.4



* $P < 0.05$

Fig. 5



* $P < 0.05$

Fig. 6