Mapping of the proinflammatory domains of MspTL of *Treponema lecithinolyticum*

Hye-Kyoung Jun,1 Hae-Ri Lee,1 Sung-Hoon Lee1 and Bong-Kyu Choi1,2

1Department of Oral Microbiology and Immunology, School of Dentistry, Seoul National University, Seoul 110-749, Republic of Korea
2Dental Research Institute, School of Dentistry, Seoul National University, Seoul 110–749, Republic of Korea

The major surface protein (MspTL) of *Treponema lecithinolyticum*, associated with periodontitis and endodontic infections, has been reported to induce proinflammatory mediators such as intercellular adhesion molecule (ICAM)-1, and interleukin (IL)-1β, IL-6 and IL-8. The purpose of this study was to examine the role of MspTL in cell adhesion/migration and to identify its proinflammatory domains. Using the human monocyctic cell line THP-1 and human dermal microvascular endothelial cells (HMEC-1), it was demonstrated that MspTL increased adhesion of monocytes to endothelial cells and transendothelial migration. To analyse the proinflammatory domains of the protein, four gene constructs covering different regions of MspTL were designed and expressed in *Escherichia coli* using the expression vector pQE-30. Histidine-tagged recombinant proteins were purified using Ni-NTA agarose and polymyxin B agarose to remove LPS contamination. Recombinant truncated polypeptides were assessed for the ability to induce ICAM-1 and proinflammatory factors in THP-1 cells by real-time RT-PCR and ELISA. Of the four polypeptides, the one spanning the N-terminal 86 amino acids significantly induced ICAM-1, IL-1β, IL-6, IL-8, tumour necrosis factor-α (TNF-α), cyclooxygenase (COX)-2, and prostaglandin E2 (PGE2). The results indicate that MspTL may induce cell adhesion and inflammation via its N-terminal region.

INTRODUCTION

Oral spirochaetes are associated with periodontal diseases and endodontic infections. They include enormously diverse *Treponema* species (Choi et al., 1994; Dewhirst et al., 2000). *Treponema lecithinolyticum* is a small saccharolytic spirochaete with phospholipase A and C activities (Wyss et al., 1999), and belongs to the phylogenetic group IV oral *Treponema* that is frequently found in periodontitis (Choi et al., 1994; Moter et al., 2006; Wyss et al., 1999). Recently, these organisms were detected in infected root canals associated with either asymptomatic or symptomatic apical periodontitis (Siqueira & Rocos, 2003). With most oral treponemes, the proportion of subjects positive for certain species or phylotypes was higher in subjects with chronic and aggressive periodontitis than in the periodontitis resistant subjects (Moter et al., 2006). This difference was pronounced for treponemes of the phylogenetic groups II and IV, and for *Treponema socranskii* and *T. lecithinolyticum*. *T. lecithinolyticum* has been reported to activate matrix metalloproteinase-2 (MMP-2) in human gingival fibroblasts and periodontal ligament (PDL) cells (Choi et al., 2001a), to induce osteoclast differentiation by a prostaglandin E2 (PGE2)-dependent mechanism (Choi et al., 2001b). The bacterium possesses the prca-prtP gene, whose product exhibits chymotrypsin-like protease activity (Correia et al., 2003).

Bacterial surface proteins promote diverse activities, including adhesion, cytotoxicity, antigenicity and host cell stimulation. Recently we reported that the major surface protein (MspTL) of *T. lecithinolyticum* induced proinflammatory factors in host cells (Lee et al., 2005). MspTL (590 amino acids, including the signal peptide of 19 amino acids) is homologous to the *Treponema maltophilia* major surface protein, MspA, with 53% identity and 59% similarity. These homologous proteins stimulate the expression of interleukin (IL)-1, IL-6 and IL-8 and intercellular adhesion molecule (ICAM)-1 in THP-1 cells, a monocytic cell line, and primary cultured PDL cells. Identification of the functional regions of bacterial surface proteins will improve our understanding of bacteria–host interactions and may help diverse eradication strategies.
The purpose of this study was to examine the role of MspTL in cell adhesion/migration and to identify its proinflammatory regions using truncated recombinant MspTL polypeptides.

METHODS

Cloning of MspTL gene fragments and purification of truncated MspTL polypeptides. MspTL gene fragments were amplified from genomic DNA of *T. leichnomyxi* (ATCC 700332) by PCR and cloned in *Escherichia coli* using the expression vector pQE-30 as described previously (Lee *et al.*, 2005). Purification of the recombinant proteins with Ni-NTA agarose and decontamination of endotoxin using polymyxin B agarose were also performed as described previously (Lee *et al.*, 2005). The protein concentration was determined by bichinchoninic acid protein assay. The primers used were described previously (Lee *et al.*, 2005). The ability of MspTL to induce monocyte transmigration across an endothelial cell monolayer was assessed using the Transwell plate system (Corning). HMEC-1 cells (5 × 10^6 cells per well) were seeded in the upper chamber of Transwell culture plates (6.5 mm Transwell inserts with 8 μm pore, Corning Costar) and cultured to confluence. Confluence of the monolayer was confirmed by microscopy. HMEC-1 cells were stimulated with 10 μg MspTL ml^{-1} in serum-free MCDB131 medium for 24 h, and in parallel, THP-1 cells were separately stimulated with 10 μg MspTL ml^{-1} in serum-free RPMI 1640 medium for 24 h. After changing the medium in the upper chamber, MspTL-stimulated THP-1 cells (1 × 10^6 cells per well) were placed on top of the HMEC-1 cells in the upper chamber and allowed to migrate through the HMEC-1 monolayer into the lower chamber at 37 °C for 20 h in a humidified atmosphere with 5% CO₂. The migration assay was also performed using MspTL-stimulated THP-1 cells/non-stimulated HMEC-1 or non-stimulated THP-1 cells/MspTL-stimulated HMEC-1. The medium was taken from the lower chamber and migrated THP-1 cells were counted under a microscope using a cell-counting haemocytometer.

Treatment of host cells with MspTL polypeptides. We used THP-1 cells to assess the biological activities of MspTL polypeptides. THP-1 cells were cultured in RPMI 1640 with 10% FBS. The cells were seeded in 35 mm cell culture dishes at a density of 1 × 10^5 cells ml^{-1} and cultured overnight. After replacing the medium with serum-free medium, the cells were stimulated with 0.16 μM of recombinant polypeptides for 12–24 h. This concentration of polypeptides was equivalent to 10 μg ml^{-1} of MspTL, which we used in our previous study (Lee *et al.*, 2005). The cells were harvested for RNA isolation and flow cytometry analysis, and the conditioned culture supernatants were stored at −70 °C for ELISA. HMEC-1 cells (1 × 10^5 cells ml^{-1}) were cultured in 35 mm cell culture dishes to confluence. After replacing the medium with the serum-free medium, the cells were stimulated with 10 μg MspTL ml^{-1} for 12 h and analysed for ICAM-1 and IL-8 mRNA expression by RT-PCR as described below. Non-stimulated cells and cells stimulated with mock extracts were used as negative controls.

Expression of ICAM-1 by flow cytometry. THP-1 cells were stimulated with recombinant MspTL polypeptides for 24 h and the expression of ICAM-1 was evaluated by flow cytometry after reacting cells with anti-human ICAM-1 antibody and subsequently with FITC-labelled IgG. We used THP-1 cells stimulated with recombinant MspTL polypeptides for 24 h and the expression of ICAM-1 was evaluated by flow cytometry after reacting cells with anti-human ICAM-1 antibody and subsequently with FITC-labelled IgG. We used THP-1 cells stimulated with recombinant MspTL polypeptides for 24 h and the expression of ICAM-1 was evaluated by flow cytometry after reacting cells with anti-human ICAM-1 antibody and subsequently with FITC-labelled IgG as described previously (Lee *et al.*, 2005).

RT-PCR and real-time RT-PCR. Total RNA was extracted from the cells stimulated with MspTL or polypeptides of MspTL using TRIzol reagent (Invitrogen). cDNA was synthesized by mixing RNA (1 μg) and Maxime RT premix (iNtRON) in a 20 μl reaction volume and incubating the mixture at 42 °C for 1 h. RT-PCR was performed to analyse the expression of ICAM-1 and IL-8 in HMEC-1 by MspTL as described previously (Lee *et al.*, 2005). For real-time RT-PCR, the cDNA (1 μl) was mixed with 10 μl SYBR Premix Ex Taq (Takara Bio) and primer pairs (0.2 pmol each) in a 20 μl reaction volume, followed by PCR for 40 cycles with 95 °C denaturation for 15 s, 60 °C annealing for 15 s and 72 °C extension for 33 s in an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). The PCR products were subjected to a melting curve analysis to verify a single amplification product. PCR without RT was performed as a negative control. The housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference in order to

http://mic.sgmjournals.org
normalize expression levels and quantify changes in gene expression between non-stimulated controls and MspTL polypeptide-stimulated cells. The expression fold change of each gene was determined from the difference in cycle numbers to reach a threshold value between control and MspTL-stimulated cells and was expressed in a log2 ratio. The genes analysed by real-time RT-PCR were ICAM-1, IL-1β, IL-6, IL-8, tumour necrosis factor-alpha (TNF-α) and cyclooxygenase (COX)-2. For the inhibition assay, the cells were pretreated with NS-398 (Sigma-Aldrich), a specific COX-2 inhibitor, for 30 min before treatment with MspTL polypeptides. The sequences of the primers for real-time RT-PCR were as follows: 5'-CATATGCCATGGGCTAACAC-3' and 5'-AGTTGTATGTCCTCATGGTG-3' for ICAM-1; 5'-AGCTGTACCCAGAGAGTCC-3' and 5'-ACCAAATGGCCGTGGTTT-3' for IL-1β; 5'-AACCTGTCCACTGGGCACA-3' and 5'-TCTGGCTCTGAAACAAAGGAT-3' for IL-6; 5'-GTGAAGGTGCAGTTTTGCCA-3' and 5'-TCTCCACAACCCTCTGCAC-3' for IL-8; 5'-CAGGGACCTCTCTAATCA-3' and 5'-AGCTGGTTATCTCTCAGCTC-3' for TNF-α; 5'-AAGCTGGGAGCCTTCTCTA-3' and 5'-GTGCTGGGCAAAGAATGCAA-3' for COX-2; and 5'-GTGGTGGACCTGACCTGC-3' and 5'-TGAGCAGGCCGCCGTCG-3' for GAPDH.

ELISA. The culture supernatants of THP-1 cells stimulated with MspTL polypeptides were assayed to determine IL-8 and PGE2 levels using ELISA kits from R&D Systems.

Statistical analysis. The statistical significance of the differences between non-stimulated and MspTL polypeptide-stimulated cells or between MspTL polypeptide and MspTL polypeptide/inhibitor-treated cells was evaluated using Student's t-test. A P value of less than 0.05 was considered significant.

RESULTS

Adhesion of monocytes on endothelial cells by MspTL
In our previous study, MspTL significantly induced ICAM-1 and IL-8 in THP-1 and PDL cells (Lee et al., 2005). ICAM-1 is a representative adhesion molecule for endothelial cells and IL-8 is a chemoattractant for various types of cells. In addition, both molecules contribute to cell adhesion and migration, and so we first analysed whether MspTL induced ICAM-1 and IL-8 in HMEC-1. As shown in Fig. 1, MspTL induced ICAM-1 and IL-8 mRNA expression. Next, we analysed the binding of monocytes to and transmigration through endothelial cell layers, using THP-1 and HMEC-1 cells. As shown in Fig. 2, MspTL significantly increased the binding of THP-1 cells to endothelial cells. The binding capacity was higher in experiments using both cell types stimulated with MspTL than in those where only one type of cell was stimulated with MspTL and the other was not stimulated.

To assess the transmigration, a chemotactic gradient was created through the endothelial cells by changing the

![Fig. 1. Induction of ICAM-1 and IL-8 mRNA expression by MspTL in HMEC-1. HMEC-1 cells (1×10⁵ cells ml⁻¹) were cultured in 35 mm cell culture dishes to confluence. After the medium was replaced with serum-free medium, the cells were stimulated with MspTL (10 µg ml⁻¹) for 12 h. The total RNA was isolated from the cells, and ICAM-1 and IL-8 mRNA expression was determined by RT-PCR. Non-stimulated cells (con) were used as a control.

![Fig. 2. Increased monocyte binding to HMEC-1 stimulated by MspTL. HMEC-1 cells were cultured to confluence and stimulated with MspTL (10 µg ml⁻¹) for 12 h. THP-1 cells were stimulated with MspTL (10 µg ml⁻¹) for 12 h were placed on the HMEC-1 and allowed to bind for 6 (white), 12 (hatched) and 24 h (grey) at 37 °C. Non-adherent monocytes were washed off and the remaining total cells were fixed with ethanol and stained with 0.1% methylene blue. After washing the unbound dye, the stained methylene blue was released using 0.1 M HCl and A₆₅₀ was measured. The value of the binding of non-stimulated THP-1 cells to non-stimulated HMEC-1 represents the basal binding. NS, non-stimulated; S, stimulated with MspTL; *, statistically significant (P<0.05) differences in cell adhesion compared to control values using non-stimulated THP-1 cells and HMEC-1.](image-url)
medium of HMEC-1 stimulated with MspTL only in the upper chamber before adding THP-1 cells. MspTL significantly increased transendothelial migration of THP-1 cells compared to non-stimulated control cells \((P<0.05)\). The migrated cell number was significantly greater in the combination of stimulated HMEC-1 and non-stimulated THP-1 cells \((30975 \pm 6682 \text{ cells})\) than in the combination of non-stimulated HMEC-1 and stimulated THP-1 cells \((16725 \pm 106 \text{ cells})\), because of a chemotactic gradient in the former case. The migrated cell number was highest in experiments using both cell types stimulated with MspTL \((39450 \pm 7990 \text{ cells})\), while the control value using non-stimulated THP-1 cells and HMEC-1 was 14525 ± 742 cells.

Expression of partial MspTL polypeptides

Truncated recombinant MspTL polypeptides were produced to determine the functional regions of MspTL with regard to host cell stimulation. MspTL and its partial polypeptides were successfully expressed in \(E. coli\) and the recombinant proteins were detected at the position of the predicted molecular masses when analysed by SDS-PAGE (Fig. 3).

Induction of ICAM-1 and IL-8 by MspTL polypeptides

To determine which fragments of MspTL promoted the biological activities, we analysed the ability of the truncated recombinant polypeptides to induce ICAM-1 and IL-8 in THP-1 cells. As shown in Fig. 4(a), PP1, 2 and 3 significantly induced mRNA expression of these factors in THP-1 cells as demonstrated by real-time RT-PCR. Among the polypeptides, PP1, which encompasses the first 86 amino acids of MspTL, induced ICAM-1 and IL-8 expression most significantly. PP4 exhibited minimal stimulating activity. To determine the induction at the protein level, we evaluated ICAM-1 expression by flow cytometry and IL-8 level by ELISA. As shown in Fig. 4(b), PP1 induced ICAM-1 and IL-8 expression most significantly and the level was equivalent to or higher than that of MspTL-induced cells. The dose-dependent induction of ICAM-1 expression by PP1 is shown in Fig. 4(c).

Induction of proinflammatory factors by PP1

Since PP1 was the most potent inducer for ICAM-1 and IL-8, we further analysed its ability to modulate other inflammatory factors. As shown in Fig. 5, PP1 significantly upregulated mRNA of IL-1\(\beta\), TNF-\(\alpha\), IL-6 and COX-2 in THP-1 cells.

Induction of PGE\(\_2\) by PP1

PGE\(\_2\) is an important inflammatory factor. Since MspTL and PP1 induced COX-2 mRNA expression, we tested for their ability to induce PGE\(\_2\), a COX-2 product. As shown in Fig. 6, MspTL and PP1 significantly induced PGE\(\_2\) in THP-1 cells. PGE\(\_2\) levels were reduced to control levels when the cells were pretreated with NS-398, a specific COX-2 inhibitor, before treatment with MspTL or PP1.

DISCUSSION

Information about biologically active sites of bacterial surface proteins is important for understanding pathogenesis and will help to deduce conserved sequences and structural elements that can be used to develop eradication strategies. In our previous study, we showed that \(T. lecithinolyticum\) major surface protein, MspTL, stimulated proinflammatory mediators including ICAM-1, IL-1\(\beta\), IL-6 and IL-8 in host cell types such as monocytes and fibroblasts (Lee et al., 2005). In additional experiments, we observed that MspTL-induced ICAM-1 expression accounted for more than 80% of that induced by the equivalent amounts of \(T. lecithinolyticum\) whole cells or lysates. Therefore, we assume that MspTL is a main player of host cell stimulation with respect to ICAM-1 induction. In this study, we further characterized MspTL with regard to its role in monocyte adhesion and transepithelial migration, and determined the proinflammatory regions using truncated recombinant MspTL polypeptides.
The interaction between monocytes and the endothelium is a key process in many inflammatory diseases, including periodontitis. Since MspTL induced IL-8, a classical chemokine, and ICAM-1, an adhesion molecule, in both monocytes and endothelial cells, we examined monocyte–endothelial cells interaction. MspTL induced monocyte adhesion to endothelial cells and transendothelial migration. These results suggest that MspTL may play an important role in the initial infiltration of monocytes into the periodontal tissues in periodontitis. Fimbriae of Porphyromonas gingivalis, a periodontal pathogen with strong proteolytic activities, have been reported to induce human monocyte adhesion to endothelial cells through the interaction between CD11b/CD18 and ICAM-1 (Harokopakis et al., 2006), depending on Toll-like receptor 2, Rac1 and phosphatidylinositol 3-kinase.

To identify regions critical to MspTL function we designed partial polypeptides of MspTL and evaluated their ability to induce inflammatory host factors. The N-terminal region of MspTL (86 amino acids) exerted a biological activity equivalent to the whole protein in terms of inducing proinflammatory mediators like IL-1β, TNF-α, IL-6, IL-8 and PGE2. Prediction of protein subcellular localization analysed using the program PSORTb (Gardy et al., 2005) resulted in the localization of MspTL in the outer...
membrane with a likelihood of 94.9%. Predicted secondary structure of MspTL using the programs PSIPRED (Jones, 1999) and DSSP (Kabsch & Sander, 1983) revealed that most of the protein is composed of β-strands and loop regions. The N-terminal 86 amino acids of MspTL form the structure of three β-sheet membrane-spanning segments with two surface-exposed loops and two periplasmic short segments, when analysed with the program PRED-TMBB (Bagos et al., 2005) predicting the topology of β-barrel outer-membrane proteins. Therefore, the N terminus could be thought to come into contact with the host cells via the surface-exposed loops.

The major surface protein, 53 kDa Msp, of *Treponema denticola*, the most intensively studied oral treponeme, is well characterized and regarded as an important virulence factor. It is an adhesin that binds to host cells and various tissue proteins such as fibronectin, keratin, laminin, collagen, fibrinogen, hyaluronic acid and heparin (Edwards et al., 2005). Msp causes cytopathic effects, forming ion channels within lipid bilayers of epithelial cells and disrupting calcium signalling in fibroblasts (Fenno et al., 1998; Wang et al., 2001). It also triggers release of MMP-8, MMP-9, cathepsin G and elastase from neutrophils (Ding et al., 1996), suggesting its role in periodontal tissue destruction. Recently, using recombinant partial polypeptides of Msp and region-specific antibodies, Edwards et al. (2005) analysed epitopes involved in mediating adhesion and found that the N-terminal half of Msp, particularly the central variable sequence region, carries this function. Although MspTL of *T. lecithinolyticum* and Msp of *T. denticola* are not homologous proteins, they are among the most abundant proteins in these bacteria. Therefore, accumulating data on the functional motifs of such proteins in oral spirochaetes will provide ideas about what controls the pathogenesis of the bacteria.

Various inflammatory mediators are synthesized in response to periodontal pathogens and their products and contribute to the initiation and progression of periodontitis, ultimately leading to periodontal tissue destruction, including alveolar bone resorption. High levels of inflammatory mediators are detected in gingival crevicular fluid (GCF) and periodontal tissues of periodontitis patients compared to periodontally healthy individuals (Gorska *et al.*, 2003; Sakai *et al.*, 2006; Wang *et al.*, 2003). The presence of IL-1β, IL-6, TNF-α and PGE2 in GCF is correlated with clinical signs of periodontitis such as a significantly higher plaque index and gingival index, deeper pocket probing depth and greater loss of attachment (Champagne *et al.*, 2003). These factors are also potent bone-resorbing mediators that induce alveolar bone resorption, leading to tooth loss. Previously, we observed that *T. lecithinolyticum* lysates induced osteoclastogenesis in a co-culture system composed of mouse bone marrow cells and calvarya-derived osteoblastic cells, and that this process was dependent on PGE2 (Choi *et al.*, 2001b). MspTL may be the main player behind the increase of PGE2 by *T. lecithinolyticum*.

In summary, we have demonstrated the ability of MspTL to induce the expression of proinflammatory mediators involved in cell adhesion, inflammation and bone resorption. The N-terminal polypeptide of 86 aa was critical to the stimulation of host cells and the pathological effect of MspTL. We are further pursuing a fine map of the N-terminal region that will contribute to the identification of potential targets to prevent pathogenesis of the major surface protein of *T. lecithinolyticum*.

**ACKNOWLEDGEMENTS**

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2006-531-00072). H.-K. J., H.-R. L. and S.-H.L. are recipients of a scholarship from BK21 program (Craniofacial Science 21).

**REFERENCES**


Edited by: M. A. Curtis