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Porphyromonas gingivalis promotes monocyte migration by activating MMP-9

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Background and Objective: The migration of monocytes into the local environment is crucial for their maturation into macrophages or osteoclasts in the pathogenesis of periodontal disease. The objective of this study was to investigate the role and mechanisms mediated by *Porphyromonas gingivalis* in promoting the migration of monocytes by regulating MMP-9 and TIMP-1 expression.

Material and Methods: Human THP1 monocytes were treated with culture supernatant derived from *P. gingivalis* (ATCC 33277) for 24 h. Zymography, western blot analysis and quantitative PCRs were performed to analyse protein and mRNA levels of MMP-9. Protein and mRNA levels of TIMP-1 from monocytes treated with or without *P. gingivalis* were determined as well. Transwell migration assay was carried out to analyse the effect of *P. gingivalis* on the migration of human peripheral blood CD14-positive monocytes. An MMP inhibitor (GM6001) and a proteinase inhibitor (leupeptin) were used to determine the role of MMP-9 in *P. gingivalis* supernatant- and lipopolysaccharide-induced monocyte migration.

Results: In zymography and western blot, an 82 kDa band of active MMP-9 emerged in *P. gingivalis*-treated monocyte culture media in a dose-dependent manner, in addition to the MMP-9 proenzyme (92 kDa) band expressed in control cell culture media. *P. gingivalis* supernatant increased both the protein and the mRNA levels of MMP-9 and TIMP-1. *P. gingivalis* supernatant, but not its lipopolysaccharide, increased the migratory ability of CD14-positive monocytes. The increased migratory ability of *P. gingivalis*-treated monocytes was partly inhibited by leupeptin (200 μg/mL) and completely antagonized by the MMP inhibitor GM6001 (100 nM). Lipopolysaccharide of *P. gingivalis* increased protein and mRNA levels of MMP-9 in monocytes, but had no effect on the migratory ability or MMP-9 activation.

Conclusion: P. gingivalis supernatant increased the migratory ability of monocytes, in part, by increasing activation and expression of MMP-9.

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Recent case–control studies and longitudinal studies have established the association between chronic periodontal disease and cardiovascular disease (1). This relationship could be mediated by inflammatory responses and cellular events induced by periodontal pathogens (2). *Porphyromonas gingivalis*, a major periodontal pathogen, has been shown to be present in both periodontal pockets and atherosclerotic plaques (3,4).

Monocytes are a circulating white blood cell and part of the human body's immune system. Activation of immune cells by periodontal pathogens, e.g. *P. gingivalis*, is an important feature of chronic inflammatory periodontal disease. The migration and localization of monocytes has been recognized to be a crucial step in their differentiation and maturation into macrophages and osteoclasts, which play important roles in the development of local inflammation and connective tissue destruction (5,6). Previous studies have shown that *P. gingivalis* modulates adhesion, chemotaxis and migration of monocytes and their derived cells by regulating the levels of cytokines (7–10), chemoattractants (11–13), cytosolic proteins (14,15) and MMPs (16).

The migratory abilities of monocytes and derived cells are partly accomplished through the regulation of MMPs and TIMPs (17–19). The MMPs are a family of zinc-dependent endopeptidases responsible for the degradation of multiple extracellular matrix components (20). MMPs are secreted in a latent proform by multiple host structural cells and immune cells. The activation of MMPs involves the loss of a propeptide (20). The TIMPs function as inhibitors of MMPs by forming noncovalent complexes with MMPs, thus blocking the access of substrates to the MMP catalytic site (20). Numerous MMPs and TIMPs are expressed in peripheral blood monocytes (21), including MMP-9 and TIMP-1. MMP-9, also known as gelatinase-B, cleaves denatured collagen, in particular, type IV collagen, which constitutes the major component of the basement membranes (22). This cleavage helps the white blood cells that play a role in immune responses, such as monocytes, to enter and leave the blood circulation. Previous studies suggest that MMP-9 and TIMP-1 are involved in the migration and localization of monocytes and their differential cells (16,18,19,23). However, how MMPs are regulated by periodontal pathogens in monocytes and how this regulation affects the migratory ability of monocytes have not been fully elicited.

Therefore, the objective of this study was to investigate the role and mechanisms mediated by *P. gingivalis* in promoting the migration of monocytes by regulating MMP and TIMP expression. It was hypothesized that *P. gingivalis* induces monocyte migration by regulating MMP-9 and TIMP-1. Understanding how periodontal pathogens alter the function of host immune cells in the pathogenesis of periodontal disease may help to explain the association of periodontal disease with other systemic conditions, such as cardiovascular disease.

Material and methods

Growth of bacteria and collection of the culture supernatant

P. gingivalis supernatant has been used in previous studies as a stimulus to mimic the toxins released from this bacterium in the periodontal pocket (24–26). *P. gingivalis* ATCC 33277 supernatant was provided by Dr A. Progulske-Fox (University of Florida College of Dentistry, Gainesville, FL, USA). *P. gingivalis* ATCC 33277 was cultured in supplemented brain–heart infusion growth media as described previously (25). The collected supernatant was filtered twice through 2 μm membranes to remove larger cell remnants and then stored at −20°C until use. The lipopolysaccharide (LPS) of *P. gingivalis* ATCC 33277 was also provided by Dr A. Progulske-Fox. The methodology for isolation and purification of LPS from *P. gingivalis* was described in a previous paper (27).

Cell culture

The THP1 human monocytic leukemia cell line was used to study the effect of *P. gingivalis* on intact human monocytes (28). Monocytes isolated from mononuclear fractions of peripheral blood of healthy donors and commercially available human CD14-poaitive $CD14^+$) monocytes were also used in this study. Monocytes were cultured at 37°C in air containing 5% CO_2 in low glucose (1 g/L) α-minimal essential medium (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 4 mM l-glutamine, 100 U/mL penicillin, 50 μg/mL gentamicin and 2.5 μg/mL fungizone.

The THP1 cells were cultured with *P. gingivalis* supernatant or LPS. Monocytes were collected from the culture flask, centrifuged, and resuspended in serum-free α-MEM at a concentration of 50,000 cells/mL. The cells were then distributed in six-well culture plates and cultured for 24 h with *P. gingivalis* supernatant (0.5–5% v/v) and *P. gingivalis* LPS (100 μg/mL). Cells cultured in serum-free medium were used as experimental controls. The conditioned culture media were collected and concentrated for zymography and western blot analysis.

Gelatin zymography

The conditioned THP1 media were mixed with nonreducing loading buffer and resolved at 150 V in 10% sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels copolymerized with 1 mg/mL gelatin. The gels were then washed with a washing solution containing 2.5% Triton X-100 for three 20 min cycles. The gels were then incubated in fresh developing buffer containing 50 mM Tris (pH 7.5), 3 mM NaN_3, 5 mM $CaCl_2$ and 1 μM $ZnCl_2$ at 37°C overnight and stained with Coomassie blue to visualize the proteolytic bands. The experiment was repeated three times.

Western blot

An equal amount of total protein of untreated and *P. gingivalis*-treated cell culture media was resolved in 10% SDS–PAGE gels at 150 V. The proteins on the gels were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) at 0.3 A for 1 h using blotting buffer [25 mM Tris–HCl (pH 8.3), 192 mM lycine and 10% methanol[. The membranes were then incubated in 5% milk in phosphate-buffered saline solution with 0.1% Tween-20 (pH 7.4) for 1 h to block nonspecific binding. The membranes were then incubated with primary antibodies at 4°C overnight.

Antibodies used in this study were monoclonal anti-MMP-9 (1:1000 dilution; Fitzgerald, Concord, MA, USA) and polyclonal antibody 2315 (5 μg/mL; a gift from Dr L. J. Windsor, Indiana University School of Dentistry, Indianapolis, IN, USA) for TIMP-1. The membranes were then washed three times with phosphate-buffered saline with Tween and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Chemiluminescence was detected by an ECL-Plus kit (Perkin-Elmer, Foster City, CA, USA). The western blot analyses were repeated three to five times.

Real-time PCR

Total RNA was isolated from cultured THP1 cells with TRIzol reagent (Invitrogen), following the manufacturer's protocol. Total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit. Real-time PCR was performed with the TaqMan Gene Expression Assay and was normalized against 18S RNA using an ABI 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA). The primers Hs00234579_ml and Hs171558_ml were used for the detection of MMP-9 and TIMP-1 expression, respectively. The experiments were repeated three or more times.

Cell migration assay

Commercially available human $CD14^+$ peripherial monocytes were used for monocyte migration studies because THP1 cells do no attach to the culture wells. The monocyte migration assay was performed using modified Boyden Chamber methods as previously described (29). Briefly, peripheral blood $CD14^+$ monocytes (Lonza, Allendale, NJ, USA) were cultured in α-MEM with 10% fetal bovine serum and 10 ng/mL of macrophage colony-stimulating factor (M-CSF) for 5 d before being used in the transwell migration assay. Cells were collected by trypsin treatment (0.25%), centrifugation (1000*g*, 5 min), and then placed in the upper chambers of transwell plates (10^5 cells per well in 250 μL α-MEM supplemented with 10 ng/mL M-CSF). The lower chambers were filled with control media (500 μL α-MEM) and the following different treatment media: *Pg* (α-MEM + 1% *P. gingivalis* supernatant); *Pg* LPS (α-MEM + 100 μg/mL *P. gingivalis* LPS); boiled *Pg* (α-MEM + 1% denatured *P. gingivalis* supernatant); *Pg* + leupeptin (α-MEM + 1% *P. gingivalis* supernatant + 200 μg/mL leupeptin); or *Pg* + GM6001 (α-MEM + 1% *P. gingivalis* supernatant + 200 nM GM6001). The cells were cultured in 24-well transwell plates for 16 h at 37°C. The upper wells were wiped with cotton tips, then fixed with 3.7% formalin overnight. The wells were then rinsed with phosphate-buffered saline twice and stained with crystal violet for 20 min. Migrated cells were counted under four random ×40 microscope fields. The means of the cell numbers in these fields were compared between groups. The experiments were repeated three times.

Statistical analysis

All data are presented as means ± SEM. Comparison between groups was made using one-way ANOVA with the Fisher multiple comparison test. A probability value of $p < 0.05$ was considered statistically significant. All cell culture experiments were performed in triplicate on at least three separate occasions.

Results

P. gingivalis supernatant induced MMP-9 activation

A proteolytic band of approximately 92 kDa of MMP-9 was detected in the untreated THP1 conditioned media (Fig. 1A). It was observed that with the addition of 0.5, 1 and 2.5% of *P. gingivalis* supernatant in the media, the 92 kDa band weakened and a band with molecular weight of approximately 82 kDa emerged (Fig. 1A) in a dose-dependent manner. With increased concentrations of *P. gingivalis* supernatant (5%), both bands weakened, indicating either the degradation of host MMPs or cell depression by the bacterial supernatant. There was an enhanced 92 kDa band but no 82 kDa band in the *P. gingivalis* LPS-treated THP1 cell culture media. The results indicate a dose-dependent conversion of pro-MMP-9 to active MMP-9.

P. gingivalis supernatant or LPS treatment increased the protein levels of MMP-9 and TIMP-1 in monocytes

The western blot confirmed the findings in gelatin zymography. Latent MMP-9 was detected in untreated THP1 culture media, *P. gingivalis* LPS-treated THP1 conditioned media and *P. gingivalis* supernatant (0.5–2.5%)-treated THP1

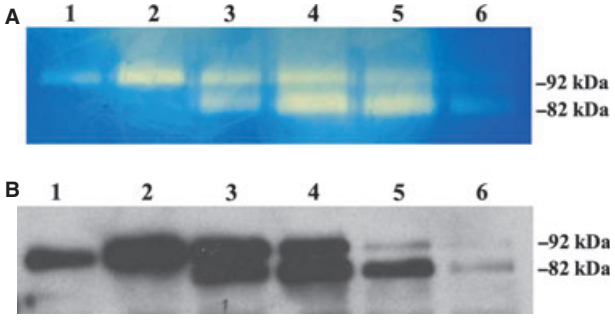

Fig. 1. MMP-9 expression and activation in THP1 monocytes cultured with or without *P. gingivalis* supernatant at different concentrations. Cell culture media were collected and concentrated for zymography (A) and western blot analysis (B) after 24 h of incubation. The lanes are as follows: (1) control THP1 culture media; (2) THP1 treated with 100 μg/mL of *P. gingivalis* LPS; (3) THP1 treated with 0.5% *P. gingivalis* supernatant; (4) THP1 treated with 1% *P. gingivalis* supernatant; (5) THP1 treated with 2.5% *P. gingivalis* supernatant; and (6) THP1 treated with 5% *P. gingivalis* supernatant.

conditioned media (Fig. 1B). The active form of MMP-9 (82 kDa) was only detected in *P. gingivalis* supernatant (0.5–2.5%)-treated THP1 conditioned media (Fig. 1B). Treatment with a higher concentration of *P. gingivalis* supernatant (5%) resulted in a decreased detectable amount of MMP-9 (Fig. 1). This could be the result of either the direct degradation of MMP-9 by bacterial proteinases or of host cell depression by high concentrations of bacterial supernatant. Therefore, 1% *P. gingivalis* supernatant was used in the cell migration analysis. *P. gingivalis* LPS increased the expression of latent MMP-9 (92 kDa), but not its active form (82 kDa). Both 1% *P. gingivalis* supernatant and LPS (100 μg/mL) increased the expression of TIMP-1 (Fig. 2A).

P. gingivalis supernatant or LPS treatment increased mRNA levels of MMP-9 and TIMP-1 in monocytes

The mRNA expression levels of MMP-9 and TIMP-1 from untreated THP1 cells and THP1 cells treated with *P. gingivalis* supernatant or LPS for 24 h were determined. Both treated and untreated THP1 cells were shown to express mRNA for MMP-9 (Fig. 3) and TIMP-1 (Fig. 2B). *P. gingivalis* supernatant or its purified LPS increased the mRNA expression of MMP-9 (Fig. 3) and TIMP-1 (Fig. 2B); however, the mRNA level of MMP-9 (Fig. 3) was much more increased than that of TIMP-1 (Fig. 2B) from *P. gingivalis*-treated THP1 cells.

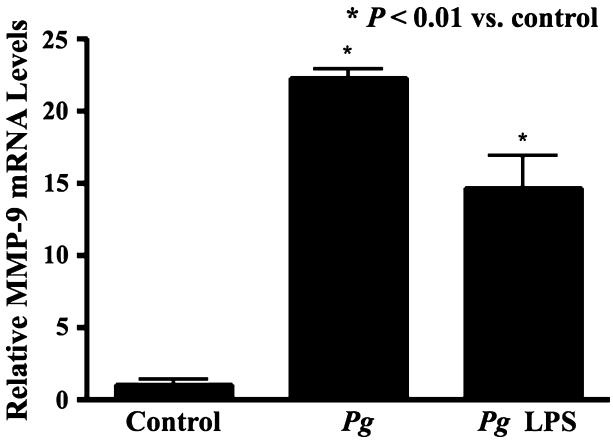

Fig. 3. Relative mRNA levels (by real-time PCR analysis) of MMP-9 from THP1 monocytes after 24 h of culture with or without *P. gingivalis* supernatant. Abbreviations: control, THP1 monocytes cultured in α-MEM only; *Pg*, THP1 monocytes treated with 1% *P. gingivalis* supernatant; and *Pg* LPS, THP1 monocytes treated with 100 μg/mL of *P. gingivalis* LPS.

P. gingivalis supernatant increased the migration of human peripheral monocytes

Treatment with 1% *P. gingivalis* supernatant increased the migration of $CD14^+$ monocytes into the lower chamber (Fig. 4). Neither *P. gingivalis* LPS nor boiled *P. gingivalis* supernatant increased the number of migrated $CD14^+$ monocytes. The cysteine and serine proteinase inhibitor leupeptin (200 μg/mL) partly reduced the cell migratory ability of the *P. gingivalis* supernatant-treated monocytes. The MMP inhibitor GM6001 (200 nM) completely antagonized the increased cell migratory ability of *P. gingivalis* supernatant-treated $CD14^+$ monocytes.

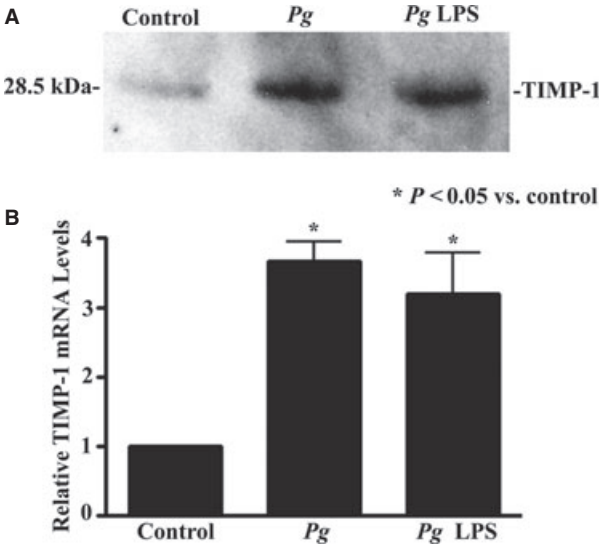

Fig. 2. Protein and relative mRNA levels of TIMP-1 from THP1 monocytes after 24 h of culture with or without *P. gingivalis* supernatant. (A) Western blot analysis. (B) Real-time PCR analysis. Abbreviations: control, THP1 monocytes cultured in α-MEM only; *Pg*, THP1 monocytes treated with 1% *P. gingivalis* supernatant; and *Pg* LPS, THP1 monocytes treated with 100 μg/mL of *P. gingivalis* LPS.

Discussion

It was revealed in our study that *P. gingivalis* supernatant increased the migratory ability of $CD14^+$ monocytes *in vitro*. This effect was accompanied by the activation of MMP-9 from the *P. gingivalis* supernatant-treated monocytes [in both THP1 cells (Fig. 1) and isolated human peripheral monocytes (data not shown)]. The increased migration of *P. gingivalis*-treated monocytes was partly reduced by a cysteine and serine proteinase inhibitor, and completely antagonized by an MMP inhibitor (Fig. 4). These results suggest that *P. gingivalis* increased the migratory ability of human monocytes through the activation and expression of MMP-9, partly mediated by the bacterial proteinases. The remaining increased migratory ability of monocytes may be induced by other protein

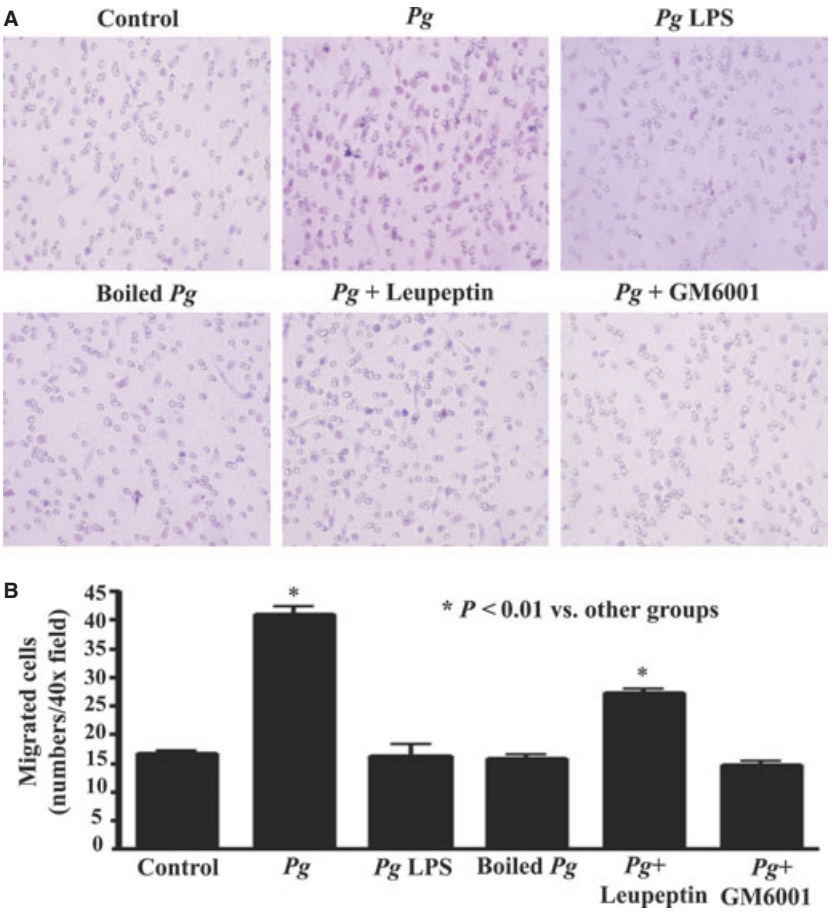

Fig. 4. Transwell migration assay for human peripheral blood $CD14^+$ monocytes. The $CD14^+$ monocytes were collected and placed in the upper chambers of transwell plates (10^5 cells per well in 250 μL α-MEM supplemented with 10 ng/mL M-CSF). The lower chambers were filled with control media (500 μL α-MEM) and the following different treatment media: *Pg*, α-MEM + 1% *P. gingivalis* supernatant; *Pg* LPS, α-MEM + 100 μg/mL *P. gingivalis* LPS; boiled *Pg*, α-MEM + 1% denatured *P. gingivalis* supernatant; *Pg* + leupeptin, α-MEM + 1% *P. gingivalis* supernatant + 200 μg/mL leupeptin; or *Pg* + GM6001, α-MEM + 1% *P. gingivalis* supernatant + 200 nM GM6001. Migrated cells were stained and counted under four random ×40 microscope fields after 16 h of incubation. The pictures were taken in ×20 fields.

components in the *P. gingivalis* supernatant, such as the fimbriae (30). This hypothesis is also indirectly supported by the fact that neither *P. gingivalis* LPS nor boiled *P. gingivalis* supernatant increased the migratory ability of the monocytes. These findings help us to understand how periodontal pathogens promote the migration of peripheral blood monocytes into the local environment of periodontitis. Furthermore, these findings, together with previous publications on how *P. gingivalis* regulates the proadhesive pathway of monocytes (30–33), provide insight into the association between *P. gingivalis* and atherosclerotic vascular disease.

This study also demonstrated that *P. gingivalis* upregulated both MMP-9 and TIMP-1 at the protein and mRNA levels. TIMP-1 is a major type of tissue inhibitor of MMPs that is synthesized by most cells (20). The balance of MMPs and TIMPs in the local environment is important in maintaining the collagen turnover rate, and disturbances of this balance may result in excessive degradation of the extracellular matrix (20). In the present study, the levels of MMP-9 and TIMP-1 were upregulated to different extents, resulting in a high MMP-9/TIMP-1 ratio. This result is in agreement with a previous study, which reported a high MMP-9/TIMP-1 ratio induced by *P. gingivalis* (live strains and LPS) from dendritic cells (16). The high MMP/TIMP ratio implies an imbalance of MMP functions in inflammatory conditions, such as periodontal disease and atherosclerotic vascular disease.

The activation of MMPs is an essential step in regulating their biological function (20). Proteolytic activation of MMPs was determined by an approximately 10 kDa reduction in molecular weight. Studies have shown that proteinases expressed by periodontal pathogens can activate latent MMPs (34,35) and may play additional roles in initiating or accelerating extracellular matrix degradation in periodontal tissues (36). Pro-MMP-1 and pro-MMP-8 can be activated by supra- and subgingival plaque extracts (37). Pro-MMP-9 can be activated by bacteria and their outer membrane extracts and cell suspension (22). Pro-MMP-2 can be activated by gingival crevicular fluid and *P. gingivalis* supernatant (38,39). *In vitro*, *P. gingivalis* proteinases can activate latent MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9, as well as catalysing the superactivation of MMP-1 by MMP-3 (34,35,38,40). Activation of these MMPs has been demonstrated to result from an initial hydrolysis within the propeptide of the MMPs. For the first time, our study demonstrates that *P. gingivalis* activates MMP-9 expressed in monocytes (Fig. 1). This activation of MMP-9, in part, contributes to the increased migratory ability of human monocytes. Activated forms of MMP-9 were detected in differentiated monocytes and were suggested to play an important role in promoting monocyte invasiveness and their differentiation into macrophages (23).

Previous studies have shown that *P. gingivalis* and its components regulate the levels of cytokines from immune cells by different mechanisms (41,42). *P. gingivalis* fimbrial protein molecules regulate monocyte chemoattractant protein-1 expression in endothelial cells (11–13). These

molecules may play an important role in structurally facilitating the attachment of bacterial protein to host cells, such as erythrocytes and monocytes (30,31,43). Live *P. gingivalis* and its LPS or fimbrillin induce different cytokines and chemokines by mouse macrophages through different ligand pathways (42,44). *P. gingivalis* LPS also induces calprotectin (a cytosolic protein) and multiple co-stimulatory molecules in human monocytes (14,15,45), as well as upregulating MMP-9 and TIMP-1 expression in human dendritic cells (16). Our study suggests that *P. gingivalis* LPS regulates MMP-9 and TIMP-1 expression in monocytes, but the *P. gingivalis* proteinases may play a crucial role in the activation of MMP-9.

Cysteine proteinases (gingipains) account for the major proteolytic activity of *P. gingivalis*. They play various roles in the pathogenesis of periodontal disease, including the development of edema, neutrophil infiltration and bleeding (46). Gingipains exhibit enzymatic activity against many host proteins, such as immunoglobulins, extracellular matrix proteins, iron-transporting factors and inhibitors, and proteins involved in the coagulation, complement and kallikrein–kinin cascades, as well as host MMPs and their activators (25,34,35,46,47). Gingipains also regulate monocyte chemoattractant protein-1 expression in endothelial cells (48). Our findings suggest that gingipains may increase the migratory ability of monoctyes by activating MMP-9. Further studies are indicated to identify the role of gingipains in regulating the localization and maturation of monocytes.

In summary, the findings of this study suggest that activation of MMP-9 is a crucial step in promoting monocyte migration induced by *P. gingivalis*. This study provided insights into the role and mechanisms of the periodontal pathogen *P. gingivalis* in the development of periodontal disease and its systemic complications.

Acknowledgements

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