Anti-inflammatory Effects of PMX205 in Mouse Macrophage Periodontitis Model

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ABSTRACT

Background: C5areceptor antagonist PMX205 is a synthetic hexapeptide capable of blocking C5a-C5a receptor (C5aR) axis by simulating C5a active C-terminal amino acid residues. This hexapeptide presents good anti-inflammatory effects in a myriad inflammation models. The anti-inflammatory effect of PMX205 on periodontitis is yet to be fully fathomed. Objective: To examine the anti-inflammatory effects of PMX205 on RAW264.7 murine macrophages exposed to gingipain extracts and Porphyromonas gingivalis (P. gingivalis). Methods: MTT assay was carried out so as to specify the cytotoxicity of PMX205. RAW264.7 cells were co-cultured in vitro with gingipain extracts or P. gingivalis to simulate the periodontitis inflammatory milieu. Real-time quantitative PCR, ELISA and Griess assay were performed in order to detect tumor necrosis factor-α (TNF-α), IL-6, IL-23, nitric oxide (NO), IL-10, transforming growth factor-β1 (TGF-β1), and arginase-1 (Arg-1). Furthermore, phagocytosis assay was done to evaluate the phagocytic capacity of RAW 264.7 cells. Finally, western blot analysis was conducted to evaluate myeloid differentiation factor 88 (MyD88). Results: PMX205 increased the expression levels of bacteriostatic substances (NO and IL-23) and anti-inflammatory cytokines (TGF-β1, IL-10 and Arg-1); however, it reduced the expression levels of proinflammatory cytokines TNF-α and IL-6 once RAW 264.7 macrophages were stimulated via gingipain extracts or P. gingivalis. In addition, PMX205 promoted the macrophage phagocytosis and down-regulated protein expression of MyD88. Conclusion: PMX205 has recognizable anti-inflammatory effects in RAW 264.7 cell inflammation model, a finding which probably opens doors to future investigations on new targets for the prevention and treatment of chronic periodontitis.


Keywords: Gingipains, Macrophage, Porphyromonas Gingivalis, PMX205, Polarization

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INTRODUCTION

The keystone-pathogen hypothesis on the etiology of periodontitis is an untrodden area to which much attention has been drawn over the recent years. In this view, Porphyromonas gingivalis (P. gingivalis) is considered as a keystone pathogen in the pathogenic progression of periodontitis (1,2). Gingipain, a major virulence factor of P. gingivalis, exhibits the activity of C5-converting enzyme which, in turn, generates a large amount of C5a that can trigger C5a-C5a receptor (C5aR) signaling pathway, and form a crosstalk with lipopolysaccharide (LPS) (3,4). The crosstalk impairs the periodontal tissue immunity inflammation, perturbing the ecologically-balanced biofilm associated with periodontal tissue homeostasis. Consequently, overt inflammation and a self-perpetuating pathogenic cycle occur, where dysbiosis and inflammation reinforce each other through forming a positive feedback loop. Eventually, the subversion of host immune responses culminates in the destruction of periodontal connective tissue and alveolar bone resorption (5). Accordingly, it is highly indispensable to develop a safe local drug for periodontitis. Among other therapeutic approaches to inflammatory disease, mention can be made of C5aR targeting of low-molecular-weight peptidomimetic antagonists (6). PMX205, a synthetic cyclic hexapeptide, is a low-molecular-weight C5aR antagonist, which can block C5a-C5aR axis by simulating C5a active C-terminal amino acid residues and exerting good anti-inflammatory effects (7-11), hence suitable for the treatment of periodontitis.

Macrophages, important parts of the innate immunity, play a crucial part in phagocytosis and periodontal immune inflammatory responses. Macrophages display robust plasticity and can be effective under external microenvironment changes which turn a variety of reactions and differentiations into M1 or M2 macrophages (12). M1 macrophages augment the production of IL-1, IL-6, IL-12, IL-23, tumor necrosis factor-α (TNF-α), reactive oxygen intermediates (ROI) and nitric oxide (NO). M2 phenotype is characterized with low NO production, increased generation of anti-inflammatory cytokines IL-10, transforming growth factor-β1 (TGF-β1) and arginase-1 (Arg-1). TNF-α, IL-1β and IL-6 are co-stimulated to mediate the destruction of periodontal tissues (13,14). Ligation of Toll-like receptors (TLRs) on the macrophage surface leads to macrophage activation by bacterial pathogen-associated molecular patterns, such as LPS (15). Widely existing in various tissues and cells is the TLR-myeloid differentiation factor 88 (MyD88)-nuclear transcription factor (NF-κB) signaling pathway which is a signaling pathway that mediates the expression of inflammatory mediators (16). The macrophages found in the gingival tissues of P. gingivalis-infected mice are predominantly M1 (CD86+) (17,18). Macrophage depletion through clodronate liposomes recognizes reduces the level of P. gingivalis infection with much less P. gingivalis-induced bone resorption (18). The effect of PMX205 on gingipain extracts- or P. gingivalis-induced macrophage polarization is yet to be reported. Therefore, the anti-inflammatory effect of PMX205 was observed in cell model experiments from the perspective of macrophage polarization.

MATERIALS AND METHODS

Cell Culture. Murine macrophage cell line RAW 264.7 was supplied by China Center for Type Culture Collection (CCTCC, Wuhan, China). Human osteoblast-like cell line MG
63 was obtained from American Type Culture Collection (ATCC, CRL-1427). Cells were cultured in DMEM (Thermo Fisher, Grand Island, NY, USA) containing 10% fetal bovine serum (Thermo Fisher) at 37°C in a 5% CO2 completely humidified incubator (SANYO, Osaka, Japan). To obtain a subculture, cells covering the bottom area of approximately 80%-90% were passed at a ratio of 1:3.

**Bacterium Culture.** *Bacterium P. gingivalis* ACTT33277 was a gift from Dr. Bei Jing (Capital Medical University, China) grown in fresh brain heart infusion (BHI, Hopebio, Qingdao, China) at 37°C under anaerobic conditions (80% N2, 10% H2, 10% CO2). Briefly, *P. gingivalis* was cultured for 5-7 days. According to the colony morphology, the culture was initially confirmed as pure. A single colony was inoculated in liquid culture medium for 48 h until logarithmic growth phase, centrifuged for bacterial collection, washed with PBS, and resuspended. UV spectrophotometer measurement was performed at 660 nm light absorption (A) value with reserved modulation.

**MTT Assay.** The logarithmic growth phase of RAW264.7 or MG 63 cells was determined, and the number of cells was adjusted to 100 μl of cell suspension (5 × 10⁴ ml⁻¹) in 96-well culture plates with PBS edge closed. The experiment was categorized into blank control group (BL) and PMX205 with different concentrations with the cells cultured in 5% CO2 at 37°C for 24, 48, and 72 h. MTT solution (20 μl of 5 mg/ml) was added to each well and incubated at 37 °C for 4 h. The liquid was carefully discarded, added with 150 μl of DMSO per well, shaken for 10 min for complete dissolution of crystals, and immediately measured at 490 nm wavelength of the light absorption (A). The relative growth rate (RGR) of the cells was calculated using the following equation: RGR (%) = (experimental group A mean/blank control group A mean) × 100%. As observed in Table 1, RGR was converted to 0-5 grade material toxicity: 0 and 1, qualified; 2, to be combined with a comprehensive evaluation of the cell morphology; >3, unqualified (19).

<table>
<thead>
<tr>
<th>RGR (%)</th>
<th>&gt;100</th>
<th>75-99</th>
<th>50-74</th>
<th>25-49</th>
<th>1-24</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
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**Real-time Quantitative PCR Analysis.** RAW 264.7 macrophages were cultured at 2.0×10⁵ cells/well in a 6-well plate for differently treated experiments, such as untreated control, gingipain extracts (4U/l, from the experimental group where acetone precipitation method extraction was employed, 20), *P. gingivalis*, or *P. gingivalis* plus PMX205 group (1μg/ml, GL Biotem, Shanghai, China). Following a 24-h culture, RAW 264.7 cells and the supernatants were collected for further experiments. Total RNA was isolated with RNeasy Mini Kit (Biomed, Beijing, China). All cDNAs were synthesized from 1 μg of the total RNA using PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan) according to the manufacturer’s recommendations. Afterwards, real-time quantitative PCR analysis assay was performed with a SYBR PrimeScript real-time quantitative PCR analysis system (Takara) and primers for mouse genes (Table 2). The conditions for real-time PCR were 30 s at 95°C, 40 cycles at 95°C for 5 s, and 60°C for 20 s. To provide analytical replicates, each gene sample was amplified in triplicate.
Data were analyzed by a comparative Ct method ($2^{-\Delta\Delta Ct}$) to calculate the relative fold changes in comparison with the untreated group. It is to be further noted that mouse β-actin gene was amplified as the internal control of the housekeeping gene.

**Table 2. Primer sequences used in this study (Takara, Japan).**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5'→3')</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>F: CATCCGTAAGACCTCTATGCAAC R: ATGGAGCCACCGATCCACA</td>
<td>171</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: GTCCTCAAGAGAGATACAGAAA R: AGCTTATCTTGATGAGGACCTTG</td>
<td>112</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: TATGGCCCAGACCCCTCACA R: GAGATAGACAAAGGATACACCCATC</td>
<td>199</td>
</tr>
<tr>
<td>IL-10</td>
<td>F: GCCAGAGCCACATGCTCTCA R: GATAAGGCTTGCAACCAAAGTAA</td>
<td>145</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>F: TACCGGCAAGGCTGAACCAA R: CGGTTCATGTCATGGGATGG</td>
<td>154</td>
</tr>
<tr>
<td>iNOS</td>
<td>F: AGCGAGGAGCGAGGTCGAA R: GAGGGGGGAATGACATGAGG</td>
<td>243</td>
</tr>
<tr>
<td>IL-23</td>
<td>F: CCCGATCCAGTGTGAAGATG R: AGGGAGGTGTGAAGTTGCTC</td>
<td>214</td>
</tr>
<tr>
<td>Arg-1</td>
<td>F: GCATTCGCAAGACACATCGT R: CAATCCCCAGCTTTGCTACTTCA</td>
<td>123</td>
</tr>
</tbody>
</table>

**ELISA and Griess Assays.** Following the manufacturer’s protocol (R&D Systems, Minneapolis, MN, USA), ELISA assay was employed to determine cytokine expression at protein levels and evaluate the level of IL-10, TGF-β1, TNF-α and IL-6 in the culturesupernatant. The NO levelin the supernatant was specified via Griess reagent (Beyotime, Shanghai, China).

**Phagocytosis Assay.** RAW 264.7 macrophages were cultured at 2.0×10⁵ cells/well in a six-well plate. The following day, they were divided into four classes: blank, control, gingipain extracts and gingipain extracts+PMX205 groups. After a 24-h culture, phagocytosis assay was used to evaluate the phagocytic capacity of RAW 264.7 macrophages following the manufacturer’s protocol (BioVision, San Francisco, USA). Cell suspension (100 µl) was transferred into 900 µl of the phagocytosis assay buffer in the flow cytometry compatible vessel and immediately analyzed in the FL1 channel of flow cytometer equipped with a laser capable of excitation at 488 nm.

**Western Blot Analysis of MyD88.** Western blot analysis was done to evaluate MyD88, an important protein in the NF-κB signaling pathway. RAW 264.7 macrophages were divided into differently treated experiments, namely, untreated control group, gingipain extracts group, gingipain extracts+PMX205 group or untreated control group, *P. gingivalis* group and *P. gingivalis* +PMX205 group. Following a 24-h culture, the cells were washed three times with cold PBS, after which, 50 µl of ice-cold RIPA lysis buffer was added, and the mixture was spun at 16,000 × g for 20 min at 4°C. The supernatant was transferred to a fresh tube kept on ice. The protein sample concentration was specified through the use of Enhanced BCA Protein Assay (Beyotime, Shanghai, China). Protein samples (20 µg) were loaded, adjusted to an equal protein concentration, diluted with 5 × loading buffer (0.63 ml of Tris-HCl (0.5 M, pH 6.8), 0.42 ml of 75% glycerol,
0.125g of sodium dodecyl sulfate (SDS), 0.25ml of β-mercaptoethanol, 0.2 ml of 0.05% bromophenol blue, and 1ml of water), heated at 95°C for 5 min, and subjected to Western blot analysis. Denatured proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) initially at 90 V for 15 min, raised up to 120 V after 1 h using the Bio-Rad Trans blot apparatus. Membranes were blocked for 10 min with 3% BSA in TBST buffer (20 mM Tris/150 mM NaCl, pH 7.5, and 0.1% Tween-20). Afterwards, the membranes were incubated with the primary antibodies of MyD88 (Cell Signaling Technology, Massachusetts, USA) (1:1000) diluted overnight through gentle rocking in 3% BSA in TBST at 4°C. Subsequently, the membranes were incubated for 1 h via appropriate HRP-labeled secondary antibodies (Cell Signaling Technology) (1:5000). After three 10-min washes, immune-reactive proteins were visualized using a chemiluminescent HRP substrate (Millipore, Billerica, MA) and a VersaDoc imaging system (Bio-Rad). The band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Results are expressed as the abundance of target protein relative to β-actin (Bio-Rad).

**Statistical Analysis.** All data, presented as means ± standard deviation (SD), were statistically analysed using one-way ANOVA with a Bonferroni post-test of Prism statistical analysis software 6.0 (Graphpad Software, La Jolla, CA). P<0.01 and P<0.05 are respectively considered as significantly different and statistically significant.

**RESULTS**

**Cytotoxicity of PMX205.**

MTT results showed that the light absorption (A) and RGR of PMX205 (0.001, 0.01, 0.1, 1, and 5 μg/ml) increased with the prolonged time, and the cytotoxicity rate was 0 or 1. However, at 10 μg/ml, the observed macrophage toxicity at 24 h was statistically different from that in the BL group (P<0.05). In researching the cytotoxicity of PMX205, it is more compelling to make use of two cell kinds. In addition to RAW264.7, we selected MG63, a major human osteoblast-like cell line which is, more often than not, utilized for periodontitis research *in vitro*. PMX205 exerted a small effect on the viability of macrophages and osteoblasts in a given concentration, thereby showing good biosecurity (Figures 1 and 2).

![Figure 1. The light absorption of RAW264.7 in different groups. MTT results showed that the light absorption (A) were increased with the prolonged time. However, at 10 μg/ml, the observed macrophage toxicity at 24 h was statistically different from the BL group (P<0.05).](image-url)
Secretion Levels of Cytokines in Inflammation Model Induced by Gingipain Extracts.
Primarily established was the inflammation model with gingipain extracts which induced high IL-23 gene expression levels and NO production. Combined with PMX205 (0.1, 1, 5, and 10 µg/ml), these extracts can induce even higher gene expressions levels of IL-23, inducible nitric oxide synthase(iNOS), TGF-β1 and Arg-1. Moreover, PMX205 (1 µg/ml) can augment the protein expression levels of TGF-β1. Such observations indicate that PMX205 can regulate or balance gingipain’s role in the macrophage polarization through C5a pathway (Figure3).

Secretion Levels of Cytokines in Inflammation Model Induced by P. gingivalis.
Data showed that P. gingivalis increased gene expression level of IL-6, TNF-α and IL-10 and the protein production of IL-6, TNF-α and NO. PMX205 enhanced the effect of P. gingivalis on the gene expression level of iNOS and IL-10 along with increasing the NO production. PMX205 further weakened the effect of P. gingivalis at gene and protein levels of TNF-α and IL-6 (Figure4). It can, therefore, be inferred that PMX205 may inhibit inflammation and promote bacterial killing and tissue healing.

Macrophage Phagocytosis.
Macrophage pretreatment with gingipains extracts increased the phagocytosis of Escherichia coli comparisons with the untreated cells, an effect which was more potent when the extracts were combined with PMX205 phagocytosedEscherichia coli. Accordingly, it is deduced that PMX205 can ameliorate macrophage phagocytosis (Figure5).

Expression of MyD88.
Western blot analysis showed that in contrast to PMX205, gingipain extracts and P. gingivalis increased the protein expression of MyD88 (Figure6).
Figure 3. Expression of inflammatory cytokines after RAW 264.7 macrophages treated with gingipain extracts with or without PMX205. (a-d) Gene expressions. (e) Protein levels of TGF-β1. (f) NO product. G, gingipain extracts. These observations indicated that PMX205 can regulate or balance gingipain’s role in the macrophage polarization. Data are represented as means ± SD from three experiments. * means P<0.05, ** means P<0.01.
DISCUSSION

Periodontitis increases the risk of diabetes, atherosclerosis, aspiration pneumonia, adverse pregnancy outcomes, rheumatoid arthritis and Alzheimer's disease (5). Therefore, its prevention and treatment is highly significant for general health and the improvement of life quality.
PMX205, a low-molecular-weight C5aR antagonist, has proved highly effective as concerns reducing the inflammatory consequences of inflammatory bowel disease (8,9), Huntington's disease (10) and Alzheimer's disease in rodent models (11). To elucidate the anti-inflammatory effects of PMX205, a cell model study was performed in vitro. The presence of macrophages in localized diseased tissues is a common characteristic observed in chronic periodontitis (21). Macrophages are differentiated into M1 macrophages when stimulated by IFN-γ or LPS; they further promote inflammation and sterilization with high expression levels of iNOS, IL-23, IL-6 and TNF-α. IL-4 and IL-10 differentiate macrophages into M2 macrophages, which increase angiogenesis and tissue repair through high expressions of anti-inflammatory cytokines, namely, IL-10, TGF-β1, Arg-1 and ferroportin 1. Macrophages can reprogram their functional phenotypes according to different inflammatory microenvironments (22,23). Under certain conditions, M1 and M2 macrophages can also be transformed into each other. Macrophage phenotype change may also play a vital role in the development and outcome of the disease (24). The present study found that RAW264.7 macrophage stimulation with *P. gingivalis* can augment proinflammatory cytokines (IL-6 and TNF-α) and bactericidal NO, meaning *P. gingivalis* can induce macrophage polarization to become M1 macrophages. Other investigations have found that *P. gingivalis* generates...
significant amounts of inflammatory cytokines in naive macrophages (25), a finding recently corroborated in a ligature model of periodontal disease, which demonstrated a higher increase in M1 macrophage markers over M2 markers in the periodontal tissues of mice (17).

Figure 6. Detection of MyD88 in RAW264.7 cells via Western blot analysis. (a) Western blot illustrating the MyD88 protein levels. Loading and transfer of equal amounts of protein was confirmed by the detection of β-actin. (b) The ratio of MyD88 to β-actin. G, gingipain extracts. Pg, *P. gingivalis*. Western blot analysis showed that gingipain extracts and *P. gingivalis* all increased the protein expression of MyD88, whereas PMX205 decreased its expression. Data are represented as means ± SD from three experiments. * means P<0.05, ** means P<0.01.

Tissue repair is mostly the transformation of M1 into M2 macrophages (26). The formulation of M2 macrophage polarization in inflammatory response can provide a new opportunity for periodontitis treatment. To preclude the cell-killing effect of the host immune system, gingipain extracts exhibit C5 invertase-like activity that can convert C5 into C5a, activate C5a-C5aRaxis and complement-mediated immune inflammatory response, along with triggering or aggravating the periodontal tissue inflammatory response and alveolar bone resorption (3,4). C5aR antagonist (PMX-53) is locally injected into the mouse periodontal tissue for the pathogenesis of the C5a-C5aR axis, which not only inhibits periodontal inflammation and alveolar bone resorption but also prevents the prophylactic inflammation inhibition of alveolar bone (27). PMX-53 is a cyclic hexapeptide Ac-Phe-(Orn-Pro-Cha-Trp-Arg)cyclevith a molecular weight of 896.13. PMX205 is also a hexapeptide hydrocinnamate-(OP-(D-Cha)WR) with a molecular weight of 705.9, which is smaller than that of PMX-53. PMX205 presents better lipophilic and metabolic stabilities than PMX-53, having a major role in reducing the production of inflammatory mediators. In addition, PMX205 promotes the release of anti-inflammatory cytokines, IL-4 and IL-10, in inflammatory diseases, such as colitis (9), allergic asthma (7) and amyotrophic lateral sclerosis (28), thereby showing excellent anti-inflammatory activity and biosafety. The iNOS produces NO which mediates macrophage bactericidal action. The present study found that PMX205 can up-regulate bacteriostatic iNOS(NO) and anti-inflammatory cytokines (Arg-1 and TGF-β1) induced by gingipain extracts. Naturally, the entire *P. gingivalis* infiltrates the periodontal tissue, hence the fact that the natural condition was mimicked using *P. gingivalis* to affect macrophages and further fathom the influence of PMX205.
PMX205 can further up-regulate the bacteriostatic and anti-inflammatory cytokines and down-regulate proinflammatory cytokines, TNF-α and IL-6, induced by *P. gingivalis*. Based on these results, PMX205 can obstruct the release of proinflammatory cytokines and increase the production of bactericidal substance and anti-inflammatory cytokines by regulating macrophage polarization. Both M1 and M2 macrophages show enhanced phagocytic capacity compared with naive macrophages (29). *Escherichia coli* and other bacterial strains are often used as pathogens in phagocytosis assays. Phagocytosis assay data clearly demonstrated that macrophage pretreatment with gingipain extracts (particularly combined with PMX205 phagocytosed *Escherichia coli*) considerably increased the phagocytosis of *Escherichia coli* compared with that of untreated cells, suggesting that PMX205 can promote macrophage phagocytosis, and clear the bacterial infections.

TLR-MyD88-NF-κB signaling pathway, a critical pathway in the reaction of inflammatory disease, is an important signaling pathway widely existing in various tissues and cells and mediating the expression of inflammatory mediators. MyD88 is a cytosolic adaptor protein with a Toll-IL-1 receptor (TIR) domain at its C-terminal; this protein is also amalgamated with the IL-1R/TLR cytoplasmic TIR domain to mediate the formation of signaling complexes (30). When ligand binding occurs, the interaction between the TIR domains of IL-1R/TLR and MyD88 transmits the signal to interleukin-1 receptor-associated kinase, subsequently activating the transcription factors NF-κB and protein-1 so as to increase the proinflammatory gene expression (16). The present results showed that, unlike PMX205, gingipain extracts and *P. gingivalis* augmented the protein expression of MyD88, indicating that gingipain extracts and *P. gingivalis* promote the inflammatory response through MyD88. Additionally, PMX205-induced MyD88 degradation is a novel negative regulation mechanism for MyD88-dependent proinflammatory signaling. C5a-C5aR axis stimulates calcium-dependent intracellular Ca^{2+} signaling, and synergistically enhances weak cyclic adenosine monophosphate (cAMP) responses activated by TLR2/TLR1 through releasing large amounts of cAMP. Increased cAMP activates the biological activity of cAMP-dependent protein kinase A (PKA) in macrophages. PKA can enfeeble antibacterial function of iNOS directly or by inhibiting the activity of glycogen synthase kinase 3β (GSK3β) to reduce the cell-killing function of macrophage (3). It can therefore be concluded that PMX205 increases iNOS, a distinct hallmark of M1 macrophage, and the anti-inflammatory cytokines which are the hallmarks of M2 macrophage. The detailed anti-inflammatory mechanism of PMX205 remains unclear and requires further research.

**ACKNOWLEDGEMENTS**

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