Original

Proviral Insertion in Murine Lymphomas 2 Promotes Inflammation and Inhibits Osteogenic Differentiation of Periodontal Ligament Cells via Regulating AMPK and NF-κB Signalings

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Abstract: Periodontitis, a chronic oral inflammatory disease, induces progressive damage to periodontal ligament and leads to tooth loss. Proviral insertion in murine lymphomas 2 (PIM2) promotes activation of NF-κB signaling, and functions as negative regulator of osteoblastogenesis. However, the role of PIM2 in periodontitis remains elusive. Firstly, cell model of periodontitis was established through incubating human periodontal ligament cells (PDLCs) with lipopolysaccharide. Lipopolysaccharide treatment decreased cell viability of PDLCs, promoted the cell apoptosis and enhanced the production of TNF-α, IL-8, and IL-6. Secondly, PIM2 expression was up-regulated in gingival tissues of patients with chronic periodontitis and lipopolysaccharide-treated PDLCs. Knockdown of PIM2 enhanced cell viability of lipopolysaccharide-treated PDLCs, and suppressed the cell apoptosis. Moreover, silence of PIM2 attenuated lipopolysaccharide-induced increase of TNF-α, IL-8, and IL-6 in PDLCs. Thirdly, the downregulated protein expression of collagen type I alpha 1 (COL1A1), osteopontin (OPN), and runt-related transcription factor 2 (RUNX2) was increased by knockdown of PIM2 in lipopolysaccharide-treated PDLCs. Lastly, interference of PIM2 up-regulated phosphorylation of AMP-activated protein kinase (AMPK), while down-regulated phosphorylation of p65 in lipopolysaccharide-treated PDLCs. In conclusion, knockdown of PIM2 exerted anti-apoptotic and anti-inflammatory effects against lipopolysaccharide-treated PDLCs, promoted the osteogenic differentiation through activation of AMPK signaling and inactivation of NF-κB signaling.

Key words: Apoptosis, Inflammation, Lipopolysaccharide, Osteogenic differentiation, PIM2

Introduction

Periodontitis, a chronic oral inflammatory disease, is characterized by tooth loosening, bleeding, gingival swelling, and alveolar bone absorption and is also the main cause of adult tooth loss. Periodontitis is generally caused by microbial infection, which could induce excessive inflammation and destroy periodontal tissue. Periodontal ligament (PDL), as connective tissue supporting the tooth nutrition, exerts osteoblast-like features. The excessive inflammation driven by microbial infection reduces the osteoblast-like features of PDL, induces progressive damage and leads to bone loss, eventually resulting in tooth loss. PDLCs, predominant cells in PDL, sense mechanical stress and participate in homeostasis and remodeling of PDL through producing dental cement and principal fibers. Excessive inflammatory response in PDLCs inhibits osteogenic differentiation and contributes to development of periodontitis. Therefore, inhibition of inflammatory response in PDLCs and promotion of osteogenic differentiation are regarded as promising therapies for the treatment of periodontitis.

Proviral insertion in murine lymphomas 2 (PIM2) functions as a serine/threonine kinase, and participates in cellular processes, including cell cycle, migration, metabolism, and apoptosis, thus regulating cancer progression. PIM2 promotes cell proliferation of multiple myeloma, and contributes to glycolysis and drug resistance of breast cancer. Inhibitors of PIM2 have been developed for the prevention of cancer progression. Previous study has shown PIM2 interacted with NLRP3 to promote lipopolysaccharide-induced NLRP3 inflammasome activation and inflammation in macrophages. Inhibition of PIM2 can suppress airway inflammation in asthmatic mice to alleviate asthma symptom. Moreover, PIM2 was up-regulated in the gingival tissue of patients with periodontitis compared to the normal tissues, and the inhibition of PIM2 repressed expression of osteoclast-related indicators in myeloma. However, the role of PIM2 in the regulation of periodontitis remains elusive.

AMP-activated protein kinase (AMPK), a regulator of intracellular ATP levels, has been shown to inhibit adipogenesis and promote the osteogenesis through regulation of osteopontin. Knockdown of AMPK increased subtypes of innate lymphoid cells and promoted expression of cytokines to worsen indices of periodontitis. Activation of AMPK reduced the production of proinflammatory cytokines and oxidative stress in gingival tissues to alleviate progression of periodontitis. PIM2 bound to AMPK α1, and promoted the phosphorylation of AMPK at Thr 467, thus leading to inactivation of AMPK signaling during aerobic glycolysis of endometrial cancer. Therefore, PIM2 might be involved in progression of periodontitis through regulation of AMPK signaling. The effects of PIM2 on cell apoptosis, inflammation and osteogenic differen-
tiation of lipopolysaccharide-treated PDLCs were then investigated in this study.

Materials and Methods

Sample collection

Total of 5 patients with chronic periodontitis and 5 healthy volunteers were recruited from The Affiliated Hospital of Zhaoqing Medical College. This study was approved by the Ethics Committee of the Affiliated Hospital of Zhaoqing Medical College (Approval no. 2020122) and in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines. All the volunteers signed written informed consents. The healthy periodontal tissues were obtained from the volunteers through crown-lengthening surgery, and the gingival samples were collected from the patients using periodontal surgery.

Cell culture and treatment

Human PDLCs were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). Cells were cultured in DMEM/F12 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum and penicillin-streptomycin (Thermo Fisher Scientific, Inc.) at 37°C. Cells were incubated with 10 μg/ml lipopolysaccharide (Sigma-aldrich Co., Ltd., St Louis, MO, USA) for 24 hours to induce periodontitis. Cells in the control group were cultured with general medium.

Cell viability and apoptosis assays

Human PDLCs were seeded into 96-well plates, and treated with lipopolysaccharide for 24 hours. Cells were then transfected with shRNA targeting PIM2 (shPIM2; GenePharma Co., Ltd., Suzhou, China) or the negative control (shNC) for another 24 hours. 10 μl MTT solution were then added into cells (5 mg/ml; Sigma-aldrich Co., Ltd.) for another 4 hours’ incubation. Dimethyl sulfoxide was then added into each well. Absorbance at 450 nm was measured by microplate reader (Bio-Rad Co., Ltd., Hercules, CA, USA). For flow cytometry, PDLCs with indicated treatment and transfection were harvested and then resuspended in binding buffer of Annexin Fitc Pi Staining Kit (Thermo Fisher Scientific, Inc.). Cells were stained with 5 µl of PI and 5 μl of annexin V (Thermo Fisher Scientific, Inc.) at 37°C. Cells were incubated with 10 μg/ml lipopolysaccharide for 24 hours. Apoptotic ratio was calculated using Image J software (National Institutes of Health, Bethesda, MD, USA).

qRT-PCR

The gingival samples and PDLCs were lysed in TRizol kit (Invitrogen Co., Ltd., Carlsbad, CA, USA), and then the RNAs were isolated. RNAs were then synthesized into cDNAs using Multiscribe™ Reverse transcription Kit (Applied Biosystems Co., Ltd., CA, USA), and the mRNA expression of PIM2, TNF-α, IL-6 and IL-8 were detected by PreTaq II kit (Takara Co., Ltd., Dalian, Liaoning, China). The mRNA expression was normalized to GAPDH through 2^−ΔΔCt method, and the primers were shown in Table 1.

ELISA

The cultured medium of PDLCs were collected, and the protein concentration was measured by BCA kit (Applygen Co., Ltd., Beijing, China). Levels of TNF-α, IL-6, and IL-8 were determined using ELISA kits (ExCell Biology, Inc., Shanghai, China).

Western blot

To investigate the role of PIM2 in periodontitis, gingival samples from patients with periodontitis and healthy people were collected. Western blot was used to incubate with PDLCs to establish cell model of periodontitis. The expression level of PIM2 was determined by western blot analysis. Western blot was used to investigate the role of PIM2 in periodontitis, gingival samples from patients with periodontitis and healthy people were collected.

Statistical analysis

All the data with at least triple replicates were expressed as mean ± SEM, and analyzed by student’s t test or one-way analysis of variance under SPSS software (IBM, Armonk, NY, USA). A p value of < 0.05 was considered as statistically significant.

Results

PIM2 was up-regulated in periodontitis

To investigate the role of PIM2 in periodontitis, gingival samples from patients with periodontitis and healthy people were collected. Western blot was used to incubate with PDLCs to establish cell model of periodontitis. The expression level of PIM2 was determined by western blot analysis. Western blot was used to investigate the role of PIM2 in periodontitis, gingival samples from patients with periodontitis and healthy people were collected.

Knockdown of PIM2 promoted cell survival of lipopolysaccharide-treated PDLCs

Lipopolysaccharide-treated PDLCs were transfected with shPIM2 to investigate the effect of PIM2 on PDLCs. Transfection with shPIM2 reduced protein expression of PIM2 in lipopolysaccharide-treated PDLCs (Fig. 2A). Cell viability of PDLCs was decreased by lipopolysaccharide treatment (Fig. 2B), while knockdown of PIM2 increased the cell viability of lipopolysaccharide-treated PDLCs (Fig. 2B). Moreover, knockdown of PIM2 attenuated lipopolysaccharide-induced increase of cell viability.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>TNF-α</td>
<td>5'-AGCCCATGTGGTACGCAAACC-3'</td>
<td>5'-GCTGGTTATCTTCAGCTCA-3'</td>
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<tr>
<td>PIM2</td>
<td>5'-TGAGTGCTCCAGTTGGCCTTCTC-3'</td>
<td>5'-CTCCAGGCTTCCTTCAACTCTC-3'</td>
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<tr>
<td>IL-6</td>
<td>5'-GTCGGTTCGATGTTAGCAAACC-3'</td>
<td>5'-CATAGGTCGATCAGGATGTTC-3'</td>
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<tr>
<td>IL-8</td>
<td>5'-CATAGGTCGATCAGGATGTTC-3'</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>5'-GCTGGTTATCTCTCAGCTCAA-3'</td>
<td>5'-AGCCCATGTTGTAGCAAACC-3'</td>
</tr>
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Table 1. Primer sequences
apoptosis in PDLCs (Fig. 2C), demonstrating that PIM2 contributed to cell apoptosis of lipopolysaccharide-treated PDLCs.

**Knockdown of PIM2 repressed inflammation of lipopolysaccharide-treated PDLCs**

Expression of TNF-α, IL-6 and IL-8 were increased in PDLCs followed by lipopolysaccharide treatment (Fig. 3A, B). However, transfection with shPIM2 reduced the production of TNF-α, IL-6 and IL-8 in lipopolysaccharide-treated PDLCs (Fig. 3A, B), revealing the

**Figure 1.** PIM2 was up-regulated in periodontitis. (A) mRNA expression of PIM2 was up-regulated in gingival samples from patients with periodontitis compared to healthy people. (B) Protein expression of PIM2 was up-regulated in gingival samples from patients with periodontitis compared to healthy people. (C) mRNA expression of PIM2 was up-regulated in lipopolysaccharide-treated PDLCs compared to the control. (D) Protein expression of PIM2 was up-regulated in lipopolysaccharide-treated PDLCs compared to the control. *** vs. healthy or control, \( p < 0.001 \).

**Figure 2.** Knockdown of PIM2 promoted cell survival of lipopolysaccharide-treated PDLCs. (A) Transfection with shPIM2 reduced protein expression of PIM2 in lipopolysaccharide-treated PDLCs. (B) Knockdown of PIM2 increased the cell viability of lipopolysaccharide-treated PDLCs. (C) Knockdown of PIM2 attenuated lipopolysaccharide-induced increase of cell apoptosis in PDLCs. *** vs. control, \( p < 0.001 \). +, +++ vs. shNC, \( p < 0.05 \), \( p < 0.001 \).
Figure 3. Knockdown of PIM2 repressed inflammation of lipopolysaccharide-treated PDLCs. (A) Transfection with shPIM2 reduced the mRNA expression of TNF-α, IL-6 and IL-8 in lipopolysaccharide-treated PDLCs. (B) Transfection with shPIM2 reduced the protein expression of TNF-α, IL-6 and IL-8 in lipopolysaccharide-treated PDLCs. *** vs. control, \( p < 0.001 \). ++, +++ vs. shNC, \( p < 0.01 \), \( p < 0.001 \).

Figure 4. Knockdown of PIM2 promoted osteogenic differentiation of lipopolysaccharide-treated PDLCs. Silence of PIM2 promoted the expression of COL1A1, OPN and RUNX2 in lipopolysaccharide-treated PDLCs. *** vs. control, \( p < 0.001 \). +, ++ vs. shNC, \( p < 0.05 \), \( p < 0.01 \).
Fan Ye et al.: PIM2 in Periodontitis

Anti-inflammatory effect of PIM2 silence in lipopolysaccharide-treated PDLCs.

Knockdown of PIM2 promoted osteogenic differentiation of lipopolysaccharide-treated PDLCs

Osteo-specific proteins, including COL1A1, OPN and RUNX2, were down-regulated in PDLCs postlipopolysaccharide treatment (Fig. 4). However, silence of PIM2 promoted the expression of COL1A1, OPN and RUNX2 in lipopolysaccharide-treated PDLCs (Fig. 4) to induce the osteogenic differentiation.

PIM2 regulated AMPK and NF-κB signaling

Protein expression of AMPK was not affected by lipopolysaccharide treatment or shPIM2 transfection (Fig. 5A). However, the phosphorylation of AMPK at Thr-172 was decreased in PDLCs postlipopolysaccharide treatment (Fig. 5A). Additionally, silence of PIM2 increased the phosphorylation of AMPK in lipopolysaccharide-treated PDLCs (Fig. 5A). Silence of PIM2 attenuated lipopolysaccharide-induced increase of p65 phosphorylation in PDLCs (Fig. 5B). These results indicated that PIM2 contributed to inactivation of AMPK and activation of NF-κB signaling in lipopolysaccharide-treated PDLCs.

Discussion

PIM family contains three members, including PIM1, PIM2, and PIM3, which have been shown to regulate cell growth and metabolism, and contribute to tumor progression. PIM2 abrogated in vitro osteoblastogenesis, and inhibition of PIM2 suppressed bone destruction and tumor growth in multiple myeloma. Moreover, PIM2 promoted the inflammatory response and activation of NLRP3 inflammasome in lipopolysaccharide-treated macrophages. This study found that knockdown of PIM2 suppressed lipopolysaccharide-induced inflammation in human PDLCs, and promoted the osteogenic differentiation to protect against periodontitis.

Previous study has shown that PIM2 was up-regulated in gingival tissue of patients with periodontitis compared to the normal tissues. Results in this study also confirmed the up-regulation of PIM2 in gingival tissue of patients with periodontitis. Periodontal pathogens, such as Porphyromonas gingivalis, secretes lipopolysaccharide to promote periodontal inflammatory response, and induce progressive damage to PDL, thus contributing to progression of periodontitis. Therefore, lipopolysaccharide-treated PDLCs were widely used as cell model of periodontitis. Here, PIM2 was also up-regulated in lipopolysaccharide-treated PDLCs. Functional assays revealed that knockdown of PIM2 increased cell viability of lipopolysaccharide-treated PDLCs and reduced the cell apoptosis, suggesting the protective effect of PIM2 silence against lipopolysaccharide-induced injury in PDLCs.

Bacterial infection induces inflammatory response in PDL through secretion of proinflammatory mediators, resulting in loss of tissue structure and function and contributing to progression of periodontitis. Suppression of periodontal inflammation is regarded as promising strat-
ergy for the prevention of periodontitis. Lipopolysaccharide-induced inflammation in macrophages has been shown to be alleviated by PIM2 knockdown. Here, knockdown of PIM2 attenuated lipopolysaccharide-induced increase of TNF-α, IL-6 and IL-8 in PDLCs, thus exerting anti-inflammatory effect in lipopolysaccharide-treated PDLCs. NF-κB pathway, playing important roles in the production of proinflammatory cytokines, is activated in periodontitis. Reducing production of TNF-α, IL-6 and IL-8 in lipopolysaccharide-treated PDLCs through inactivation of NF-κB pathway facilitated the prevention of periodontitis. PIM2 activated NF-κB signaling to promote tumorigenesis of hepatocellular carcinoma and lung adenocarcinoma. Knockdown of PIM2 repressed the phosphorylation of fructose-1, 6-bisphosphatase 1, and reduced the protein stability of NF-κB p65. Our results showed that silence of PIM2 attenuated lipopolysaccharide-induced increase of p-p65 in PDLCs, suggesting that PIM2 knockdown suppressed lipopolysaccharide-induced inflammation in PDLCs through inactivation of NF-κB signaling. Furthermore, oxidative stress is also implicated in the pathogenesis of periodontitis. PIM2 might also be involved in lipopolysaccharide-induced oxidative stress in PDLCs.

Periodontal inflammation driven by bacterial infection impaired osteogenic differentiation of PDLCs, resulting in bone loss during the development of periodontitis. AMPK signaling is essential for osteogenesis through down-regulation of growth factor independence-1 and up-regulation of OPN. Therefore, AMPK activation was considered to be a promising strategy for the alleviation of periodontitis. Inhibition of PIM2 reduced protein expression of Osterix to prevent bone loss in myeloma. Moreover, PIM2 functioned as a negative regulator of liver kinase B1 to reduce the activity of AMPK through decreasing AMPK phosphorylation at Thr 172 and increasing AMPK phosphorylation at Thr 467. Our results demonstrated that protein expression of COX-1, OPN and RUNX2 in lipopolysaccharide-treated PDLCs were increased by knockdown of PIM2, and silence of PIM2 increased phosphorylation of AMPK at Thr 172 in lipopolysaccharide-treated PDLCs. Therefore, PIM2 knockdown promoted osteogenic differentiation of PDLCs to prevent periodontitis through activation of AMPK signaling.

In summary, PIM2, up-regulated in gingival samples of patients with periodontitis and lipopolysaccharide-treated PDLCs, contributed to development of periodontitis. Silence of PIM2 suppressed lipopolysaccharide-induced inflammation in PDLCs through inactivation of NF-κB signaling and promoted the osteogenic differentiation through activation of AMPK signaling. However, the role of PIM2 in in vivo animal model of periodontitis should be investigated in further research.

Competing Interests

The authors state that there are no conflicts of interest to disclose.

Reference


